2009

Determinants Of Chloroplast Gene Expression And Applications Of Chloroplast Transformation In Lactuca Sativa And Nicotiana Tabacum

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DETERMINANTS OF CHLOROPLAST GENE EXPRESSION AND APPLICATIONS OF CHLOROPLAST TRANSFORMATION IN *LACTUCA SATIVA* AND *NICOTIANA TABACUM*

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A dissertation submitted in partial fulfillment of the requirements
for the degree Doctor of Philosophy in Biomedical Science
in the College of Medicine
at the University of Central Florida
Orlando, Florida
Summer Term 2009

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ABSTRACT

Genetic modification of plastids in the model plant tobacco (*Nicotiana tabacum*) has demonstrated that numerous foreign gene products can accumulate to high levels in this setting. Plastid biotechnology is maturing to encompass the improvement of food and feed species and the production of biopharmaceutical proteins for oral delivery necessitating development of stable transplastomic edible plants. In the interest of establishing an edible platform we have investigated the use of native and foreign regulatory elements in relation to foreign gene expression in plastids. Multiple sequence alignments of intergenic regions for 20 species of angiosperm showed that despite 95% identity in the coding region, identity in the *psbA* upstream region is 59% across all taxa examined, other gene coding regions displayed sequence identity of 80-97%, whereas the non-coding regions were 45-79% suggesting that our physical data can be extrapolated beyond the model presented.

We found that by exchanging *psbA* untranslated regions (UTRs) between *N. tabacum* and lettuce (*Lactuca sativa*), the expression of the CTB-proinsulin (CTB-Pins) monocistronic transcript declined by 84% and foreign protein accumulation was reduced by as much as 97% in mature leaves. Polyribosome association assays suggest that ribosome-free transgenic transcripts are stabilized where the native UTR is employed. RNA EMSA revealed that binding proteins interacted with *psbA* 5' UTRs in a species specific manner and the half life of the *L. sativa* 5'UTR-CTB-Pins mRNA was reduced by 3.7 fold in *N. tabacum* stromal extracts. Our data indicate that the use of

species-specific regulatory elements could lead to establishment of reproducible plastid transformation in desirable target species such as *L. sativa*.

Using transplastomic *L. sativa* for oral delivery of bioencapsulated CTB-Pins we delayed the onset of diabetes in NOD mice when retinyl acetate supplement was provided compared to untouched mice. In this 30 week study we monitored blood glucose levels and evaluated the *in vitro* suppressive capacity of regulatory T cells isolated from diabetic mice. Whether delay or prevention was achieved appeared to be a function of antigen dose as high dose resulted in a nine week delay of onset while low dose reduced the incidence of diabetes by 36%. In addition we have evaluated metabolic engineering in the *N. tabacum* model where we generated *cis*-genic lines expressing nucleus-encoded methionine pathway enzymes in plastids. Transplastomic expression of Cystathionine gamma-Synthase led to a three-fold increase in enzyme activity and a doubling of methionine content in leaves without a deleterious phenotype.

In exploring molecular mechanisms supporting gene expression in plastids and applying transplastomic technology to real human problems this work seeks address the potential of plastid biotechnology for improvement of commodity crops and production of biopharmaceuticals.

ACKNOWLEDGEMENTS

I am grateful to Dr. Henry Daniell for the opportunity to study and work in his lab at UCF, as well as to my Committee Members and Dr. Steve Ebert for their time and support. Without financial support from NIH and USDA grants generously provided to me by Dr. Daniell I would not have been able to carry out this work; for this I offer my thanks. I will always remember fondly my lab colleagues for camaraderie and advice throughout this very challenging experience and wish them all the best in the future. The dedication and patience of my future husband, Robert K. Jansen, and my beloved son, George J. Ruhlman V, have provided me with the inspiration to follow my path; for their contribution to my success I hope to convey my loving thanks through the years ahead.

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ABBREVIATIONS

aadA - Aminoglycoside 3' adenosyltransferase

atp1 – plastid ATP Synthase subunit 1

AVG - Aminoethoxyvinyl glycine hydrochloride

BADH – Betaine aldehyde dehydrogenase

BME – 2-Mercaptoethanol

bp – base pair

BSA – Bovine serum albumin

CGS – Cystathionine gamma-Synthase

CPM – Counts per minute

CSC – Cysteine Synthase Complex

CTB - Cholera toxin B subunit

CTB-Pins – Cholera toxin B subunit-human proinsulin fusion protein

DC – Dendritic cells

DTT – dithiothreitol

EDTA - ethylenediaminetetraacetic acid

EMSA – Electrophoretic mobility shift assay

g10 – Translation control region from bacteriophage T7 *gene 10*

GAD – Glutamic acid Dehydrogenase

GALT – Gut-associated lymphoid tissue

GFP – Green fluorescent protein

GSH – Glutathione

GUS – β-glucuronidase

HK – Homoserine Kinase

HRP – Horseradish Peroxidase

IGS – Intergenic regions

IR - Inverted repeat

kb – Kilobases

kDa – kiloDalton

LB – Luria-Bertani broth

LR – Lettuce regeneration media

Ls – *Lactuca sativa*

LSC – Large single copy region

MS – Murashige and Skoog basal salts

NEP – Nuclear encoded plastid localized RNA polymerase

NOD – Nonobese diabetic mouse

N-P-K – Nitrogen, phosphorus, potassium

nptII - Neomycin Phosphotransferase

Nt – *Nicotiana tabacum*

OAS-TL – O-acetylserine (thiol) Lyase

OPH – O-phosphohomoserine

PAG - DL-propargylglycine

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PEP – Plastid encoded RNA polymerase

PMSF - phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride

P*rrn*- Plastid ribosomal operon promoter

P*rrn*1 – PEP promoter element 1

P*rrn*F – Full length ribosomal operon promoter

psbA – Photosystem II core polypeptide D1

RA – Retinyl acetate

rbcL - Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit

RBP – RNA binding proteins

RBS – Ribosome binding site

rps16 – plastid ribosomal protein S16

rrn – Plastid ribosomal operon

SAM – S-adenosyl-L-methionine

SAMS – S-adenosyl-L-methionine Synthase

SAT – Serine Acetyltransferase

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SSA – Sunflower seed albumin

SSC – Small single copy region

T1D – Type 1 diabetes

TAE – Tris-acetate-EDTA

TCR – Translation control region

Teff – Effector T-cells

TLP – Total leaf protein

trc – tryptophan-β lactamase hybrid promoter

Treg – Regulatory T-cells

TS – Threonine Synthase

TSP – Total soluble protein

UTR – Untranslated region

GENERAL INTRODUCTION

Stagnation in absolute yield increases of major crops around the world is raising awareness that breeding for quantitative yield characteristics will most likely be insufficient to meet the needs of the burgeoning global population. Genetic improvement of crop species through the incorporation of novel DNA into the nuclear genome represents a powerful tool to introduce desirable agronomic characteristics (Castle et al., 2006; Ruhlman and Daniell, 2007), yet negative public sentiment partially fueled by the fear of transgene escape via pollen or seeds has hampered the acceptance of genetically modified (GM) crops in some markets. In addition, foreign protein expression in nuclear transgenic lines may be below the level required for efficacy in the field or for downstream yield.

Plastid transformation presents an attractive alternative to nuclear transformation and offers the potential to ameliorate environmental concerns. Most agronomically important species exhibit maternal inheritance of plastid genomes (Daniell, 2002; Hagemann et al., 2004) abrogating the threat of transgene escape through pollen. In maternal inheritance systems, paternal transmission of plastids is impeded during either the first pollen mitosis *via* unequal plastid distribution, or during generative or sperm cell development *via* plastid degeneration (Zhu et al., 1992; Mogensen and Rusche, 2000; Birky, 2001). The result is that the generative and sperm cells in mature pollen tend to be free of plastids. To determine if these natural mechanisms provide effective containment of transgenes in transplastomic plants investigators have utilized large scale analyses of progeny derived from cross-fertilization experiments. Two

independent studies demonstrated that while there is some degree of paternal leakage in the model *N. tabacum* cultured in vitro, the frequency was quite low. These studies estimated that transmission into the seedling cotyledons was on the order of one event in more than 10, 000, up to one in 100, 000 individuals (Ruf et al., 2007; Svab and Maliga, 2007). It is likely that the level of transmission would be lower still in the field in the absence of selective pressure in tissue culture. One study described that the plastome was heteroplasmic. Offspring that carried paternal copies contained maternal plastome as well. Following progeny (2.1 million seedlings) beyond the cotyledon stage, it was found that persistence of the paternal plastome copies was reduced to fewer than one in two million in the apical meristem (Ruf et al., 2007).

Gene silencing is not observed in plastid transformants due to site directed, single copy insertion by homologous recombination (Fagard and Vaucheret, 2000). Plastid shuttle vectors carry the gene of interest along with a gene to facilitate selection of transformed cells, often as a single integration cassette. The cassette is bordered by sequences homologous to the desired insertion site flanking regions on the native plastid genome. When the primary transformation event occurs transgenes are integrated precisely between the homologous regions, presumably in a single copy of the plastome. Depending on the site chosen for integration, intra-molecular recombination events may be predicted. Plastid transformation vectors (Guda et al., 2000) may introduce foreign sequences into the inverted repeat (IR) region of the plastome, thus the new sequence must be incorporated into both repeat regions, a phenomena referred to as copy correction. The plastome appears to exist in at least

two orientations which are present in equimolar proportions within a single plant (Palmer, 1983). The circular molecule undergoes interconversion to a dumbbell-shaped conformation that is believed to be facilitated by the presence of the IR. Concerted evolution within the IR (Kolodner and Tewari, 1975; Kolodner et al., 1976) suggests intra-molecular recombination between the repeats as there exists no report where the IR is unbalanced.

The plastome persists in all plastid differentiation types. Within mature leaf chloroplasts there may be up to 100 identical copies present (Maier et al., 2004). The mesophyll cells of a mature leaf may carry up to 100 chloroplasts with the result that plastome can comprise up to 20 % of the total cellular DNA content (Bendich, 1987). For the establishment of stable transplastomic lines homoplasmy, the iteration of the transgene throughout all copies of the plastid genome, must be accomplished. The mixed genotype is unstable and this condition, called heteroplasmy, does not persist, resolving into one state (wild type) or the other (transformed) (Maliga et al., 1993). The transformation of all copies is facilitated by culturing on non-lethal selective media. Plastids carrying the resistance marker, and in turn the cells that harbor these plastids, are preferentially maintained as plastome molecules are divided up between daughter plastids and subsequently as plastids are partitioned between daughter cells at mitosis (Maliga et al., 1975; Moller and Moller, 2005).

Foreign proteins can accumulate to high levels in transplastomic lines and are retained within the plastid envelope protecting them from degradation by host cytoplasmic proteases (Adam and Clarke, 2002). Transformation strategies have taken

advantage of the prokaryotic-type transcription and translation apparatus in plastids, which facilitates the expression of multigene operons (Quesada-Vargas et al., 2004). Co-expression of chaperones and multi-subunit complexes has contributed to improved accumulation of foreign proteins (Daniell et al., 2001; De Cosa et al., 2001; Ruiz et al., 2003). Plastid transformation technology has been implemented to confer such desirable plant traits as insect and disease resistance, salt and drought tolerance and herbicide resistance (Bock, 2007; Verma and Daniell, 2007) in the model plant *N. tabacum*. Stable plastid transformation in *Daucus carota, Gossypium hirsutum*, (Kumar et al., 2004a, 2004b), *Glycine max* (Dufourmantel et al., 2004; Dufourmantel et al., 2006) as well as encouraging progress in *Oriza sativa* (Khan and Maliga, 1999; Lee et al., 2006) and oilseed members of the Brassicaceae (Hou et al., 2003; Skarjinskaia et al., 2003) suggest the potential to engineer valuable commodity crops with superior agronomic traits using transplastomic technology.

Beyond the ability to increase the abundance and nutritional value of food sources lies the potential to express proteins of industrial interest in plant plastids. Accumulation of such diverse compounds as protein polymers (Guda et al., 2000), vaccine antigens and other clinically relevant proteins has been achieved without concomitant loss of host plant viability. Concentrations at or beyond that which would be required for feasibility in subsequent refinement procedures have been obtained (Scotti et al.; Daniell et al., 2001; Fernandez-San Millan et al., 2003; Tregoning et al., 2003; Watson et al., 2004; Koya et al., 2005; Arlen et al., 2007; Ruhlman et al., 2007; Arlen et al., 2008; McCabe et al., 2008; Zhou et al., 2008; Oey et al., 2009).

The possibility to deliver pharmaceutical proteins orally has far reaching implications. Elimination of costs related to purification, cold shipping and storage and the need for sterile injection by medical professionals make the advancement of this technology very attractive. Local production of the source crop would be an additional advantage in developing nations adding to the allure of this system as a pharmaceutical platform. To improve transmucosal absorption of antigens, internalization *via* the ganglioside M1 (GM1) receptor on the cells of the intestinal epithelia has been investigated using plastid produced cholera toxin subunit B (CTB) green fluorescent protein (GFP) fusion. Limaye et al. (2006) have demonstrated the potential of a plant based oral delivery system. Inclusion of the recognition sequence for the ubiquitous protease furin between CTB and GFP allowed for intracellular cleavage of the fusion product and subsequent transport of GFP, but not CTB, *via* the mucosal vasculature to the liver and spleen of mice fed with pulverized transplastomic *N. tabacum* leaf tissue. Findings such as these are promising and highlight the need to develop a robust platform for transplastomic expression of pharmaceutical proteins in a non-toxic edible plant.

Within the angiosperms the plastome carries approximately 120 to 130 genes and ranges in size from 120 to 180 kilobases (kb) (Sugiura, 1992). Genes encoded by the plastome include ribosomal RNAs, the full complement of transfer RNAs required for protein synthesis and proteins involved plastid function, yet the plastid expression system is far from autonomous. Of the estimated 3000 or more proteins found in the higher plant chloroplast (Martin et al., 2002; Colas des Francs-Small et al., 2004), only a

small fraction are encoded by the plastome (Shimada and Sugiura, 1991). The bulk of the plastid proteome is nuclear encoded, translated on cytosolic ribosomes and subsequently translocated across the chloroplast envelopes (Zerges, 2000). Among these imported proteins are various factors that are required for transcription and translation in plastids.

While the majority of plastid encoded proteins are involved in photosynthesis, or in assembly of the translational apparatus to express photosynthetic constituents, a considerable proportion of the imported protein complement comprises metabolic enzymes (Lunn, 2007; Ruhlman and Daniell, 2007). Synthesis of amino acids, fatty acids, isoprenoids, flavanoids, vitamins; essentially the constituents that make plants nutritionally valuable, is executed entirely or in part within plastids. Transgenic strategies aimed at nutritional enhancement of commodity crops that lack particular desirable metabolic products have begun take advantage of plastid biotechnology to tailor metabolic enzyme expression for the accumulation of previously absent nutritional components (Wurbs et al., 2007), potentially freeing end product accumulation from nuclear control mechanisms.

A limitation in the extension of transplastomic technology beyond the established model system of *N. tabacum* may be the implementation of foreign regulatory elements such as promoter and untranslated mRNA sequences (UTR) to drive expression of selectable marker/and or gene of interest. The availability of plastid genome sequences in the public databases is a valuable tool for design of plastome insertion sites and choice of regulatory elements. Development of efficient homology based plastid

transformation and expression systems will require familiarity with intergenic regions (IGS) and regulatory sequence elements. Reports indicate that plastid regulatory elements for transcription (Lerbs-Mache, 2000) and translation (Kramzar et al., 2006) may be highly species-specific. Furthermore, IGS and introns of plastid genomes have been shown to have a high degree of variability. Where high sequence identity is required, such as the flanking regions that direct homologous recombination in plastid transformation experiments, species-specific sequences may be superior.

Rationale and Approach

Nuclear transgenic approaches to engineer plants that stably express vaccine antigens and therapeutic proteins for oral delivery have met with limited success in terms of sufficient accumulation of product. This fact, coupled with concerns about transgene containment, particularly where there is potential for genetic contamination of food crops, leads to the consideration of an alternative platform. Plastid transformation technology has produced *N. tabacum* lines that accumulate therapeutic proteins in abundance. Harnessing the potential of plastid transformation for oral delivery of pharmaceutical products will require the extension of the technology to include non-toxic, edible plants. The success of this approach will demand a robust platform, wherein foreign protein will accumulate to physiologically relevant levels such that oral delivery of tolerable amounts of minimally processed plant material will achieve the desired effect.

Our main objective in this research is to explore mechanisms that underlie foreign protein accumulation in transplastomic lines using native or foreign elements for transcription and translation. We will generate transplastomic *L. sativa* that express an autoantigen, the CTB-human proinsulin fusion protein (CTB-Pins) and investigate the use of this system as a platform for oral delivery of an autoantigen. We will carry out an *in vivo* efficacy study utilizing the murine model of type one diabetes (T1D) to determine if oral delivery of transplastomic *L. sativa* expressing CTB-Pins can delay the onset of disease. We will also conduct an investigation aimed at the production of transplastomic plants that accumulate sulfur amino acids by expressing native metabolic enzymes in plastids to evaluate the efficacy of this approach in the model system *N. tabacum.*

DETERMINANTS OF PLASTID GENE EXPRESSION

Introduction

Over the expanse of time since the endosymbiotic events that led to the establishment of plant organelles, plant cells have evolved elaborate mechanisms to coordinate the expression of plastid genes with the changing developmental and functional requirements of the cell. Once autonomous organisms, plastids of extant plant species possess genomes that are greatly reduced in comparison to the autotrophic cyanobacterium species thought to represent their free-living ancestors (McFadden, 1999; Raven and Allen, 2003). The genes coding for the bulk of the plastid proteome have been relocated to the nucleus including many characterized proteins involved in the transcription and translation of plastid-encoded genes (Martin et al., 2002; Kleine et al., 2008). In addition to the nuclear encoded, plastid localized RNA polymerase (NEP), the nucleus harbors and controls the expression of a suite of sigma factors required for the active transcription of photosynthetic genes by the plastid encoded RNA polymerase (PEP), with PEP itself being transcribed by NEP (Allison et al., 1996; Hess and Borner, 1999). Nuclear control of translation of plastid mRNA is exerted through the activities of numerous plastid-localized RNA binding proteins (RBPs). RBPs appear to have a tight affinity for their cognate sequences in plastid mRNAs and studies have demonstrated that their interactions are specific for particular genes (Alexander et al., 1998; Nakamura et al., 1999; Shen et al., 2001; Miyamoto et al., 2002; Meierhoff et al., 2003; Schmitz-Linneweber et al., 2005; 2006).

Plastid gene order and content is well conserved across diverse genera of land plants (Raubeson and Jansen, 2004; Bock and Timmis, 2008). Many plastid genes are transcribed as polycistronic pre-mRNAs from operons which generally contain related functionalities, while other genes, including tRNAs and protein coding genes, are present as single transcription units (Sugiura, 1992; Mullet, 1993). Plastid mRNAs, expressed as mono- or polycistrons, contain 5' and 3' UTRs. Detailed analyses have demonstrated that within these UTRs lie so-called *cis* elements, often forming secondary structures, which facilitate the recruitment of nuclear encoded RBPs (Yang et al., 1995; Hirose and Sugiura, 1996; Klaff et al., 1997; Alexander et al., 1998; Bollenbach and Stern, 2003; Zou et al., 2003; Merhige et al., 2005; Bollenbach et al., 2009). RBPs display an array of functions including processing of polycistronic transcription units, RNA maturation and editing, transcript stability and turnover and the recruitment of additional protein factors involved in initiation of translation in response to the demands of the cell (Nickelsen, 2003). In contrast to the high level of conservation found within the regions coding for protein and ribosomal RNAs, intergenic and untranslated regions appear to be highly variable in plastids (Daniell et al., 2006; Saski et al., 2007; Timme et al., 2007).

Plastid transformation strategies have utilized both endogenous and foreign regulatory elements to facilitate high levels of foreign protein accumulation. Hybrid systems comprising a modified *N. tabacum* ribosomal operon promoter (P*rrn*) in conjunction with a translational control region (TCR) derived from the *N. tabacum* plastid-encoded *rbcL* gene or from bacteriophage T7 gene 10 (g10) to express foreign

protein have been utilized in numerous species (Kuroda and Maliga, 2001; Ruhlman et al., 2007). Another approach incorporates the native *psbA* 5' and 3' UTRs into transformation constructs (Verma et al., 2008). The potential of *psbA* 5' UTR stems from the role of its gene product in plastids. Photosystem II core protein D1 is a polytopic thylakoid membrane constituent with five membrane-spanning helices encoded by the plastid *psbA* gene (Marder et al., 1987). Expression of D1 is predominantly regulated at the level of translation and requires the participation of RBPs imported into plastids post-translationally from the cytoplasm (Hirose and Sugiura, 1996; Alexander et al., 1998; Nakamura et al., 2001). Photosystem II is highly susceptible to excessive light and the primary target of the damage is D1. If the core protein is not efficiently removed and replaced the result is impairment of electron transport, known as photoinhibition (Yamamoto, 2001). It is this cycle of damage and replacement that makes the 5' UTR of *psbA* attractive as a tool to enhance the level of foreign protein accumulation in transplastomic lines. The implementation of endogenous *psbA* regulatory elements has facilitated the generation of transplastomic *N. tabacum* lines with enhanced expression of pharmaceutical proteins (Singh et al., 2009) and generated transplastomic plants with improved agronomic characteristics (Verma et al., 2008).

While *N. tabacum* promoters and translation elements have been used to generate transplastomic plants from diverse genera with varying degrees of success in terms of transformation efficiency and foreign protein accumulation, the bulk of these reports describe the expression of selectable markers or reporter gene products (Table

1). Recently, our interest in developing a robust platform for plant-derived oral delivery of human therapeutic proteins and vaccine antigens has focused our attention on *L. sativa* as an amenable vehicle (Lelivelt et al., 2005; Kanamoto et al., 2006; Ruhlman et al., 2007). *Lactuca sativa* has provided a practical alternative to *N. tabacum* for plastid transformation experiments in that regeneration by somatic organogenesis is possible from bombarded leaf explants. This feature combined with its relatively quick cultivation time allows for the production and harvest of transplastomic biomass in as little as six months. In the course of our endeavor to develop transplastomic plants expressing maximal levels of human therapeutic proteins we have established several lines of *N. tabacum* and *L. sativa* expressing the CTB-Pins from endogenous or heterologous elements. We have used this suite of transplastomic lines along with wild type plants to investigate RNA-protein interaction, foreign transcript accumulation and polyribosome association (polysome assay), and foreign protein accumulation and turnover. In order to extend our observations beyond the scope of this model system we have also taken a bioinformatic approach to evaluate nucleotide variability in the regions upstream of plastid genes that comprise promoters and UTRs. We examined coding and non-coding sequence across 20 crop species representing most major clades of angiosperms including 4 grasses and 3 legumes. Our findings indicate that species-specific optimization of plastid transformation constructs will have a significant impact on foreign protein accumulation.

Table 1: Transplastomic species generated using heterologous regulatory elements

Species	Selectable marker	Promoter / 5'UTR	3' UTR	Gene of interest	Promoter/ 5'UTR	3' UTR	Reference
L. sativa	aadA	Nt Prrn1/ Nt rbcL RBS	Nt psbA	GFP	Nt Prrn/ Nt rbcL UTR	rrnB	Lelivelt et al., 2005
L. sativa	aadA	Nt PrrnF/RBS	Nt psbA	GFP	Nt psbA	Nt rps16	Kanamoto et al., 2006
L. sativa	aadA	Nt PrrnF/RBS	Nt psbA	CTB-Pins	Nt PrrnF/g10	Nt rps16	Ruhlman et al., 2007
S. tuberosum	aadA aadA	Nt Prrn/RBS trc g10	Nt psbA rrnB	GFP GFP	trc/g10 Nt Prrn/RBS	rrnB Nt psbA	Nguyen et al., 2005
S. tuberosum	aadA aadA	Nt Prrn1 / Nt rbcL RBS Nt psbA	Nt psbA Nt psbA	NONE EXPRESSED GFP	NONE Nt Prrn/RBS	NONE Nt rps16	Sidorov et al., 1999
G. max	aadA	Nt Prrn1 / Nt rbcL RBS	Nt psbA	NONE EXPRESSED	NONE	NONE	Dufourmantel et al., 2004
G. max	aadA	Nt Prrn1 / Nt rbcL RBS	Nt psbA	Cry1Ab	Nt PrrnF/g10	Nt rbcL	Dufourmantel et al., 2005
S. lycopersicum	aadA	Nt Prrn1 / Nt rbcL RBS	Nt psbA	NONE EXPRESSED	NONE	NONE	Ruf et al., 2001
S. lycopersicum	aadA	Nt Prrn1 / Nt rbcL RBS	Nt psbA	crtY, carRA	Nt atpI	Nt rps19	Wurbs et al., 2007
O. sativa	aadA	Nt PrrnF/g10	na	GFP	na	Nt psbA	Sarwar & Maliga 1999
O. sativa	aadA	Nt PrrnF/ RBS	na	GFP	RBS	Nt psbA	Lee et al., 2006

Species	Selectable marker	Promoter / 5'UTR	3' UTR	Gene of interest	Promoter/ 5'UTR	3' UTR	Reference
A. thaliana	aadA	Nt Prrn1/Nt rbcL RBS	Nt psbA	NONE EXPRESSED	NONE	NONE	Sikdar et al., 1998
Brs. napus	aadA	Nt Prrn1/Nt rbcL RBS	Nt psbA	cry1Aa10	PrrnF/native	*O. sativa* psbA	Hou et al., 2003
Lesquerella fendleri	aadA	Nt Prrn/Nt rbcL TCR	na	GFP	na	Nt psbA	Skarjinskaia et al., 2003
Brs. olearacea (Capitata)	aadA	Nt Prrn1/Nt rbcL RBS	Nt psbA	Cry1Ab	Nt Prrn1/Nt rbcL RBS	Nt psbA	Liu et al., 2007
Brs. olearacea (Botrytis)	aadA	Nt Prrn1/Nt rbcL RBS	Nt psbA	NONE EXPRESSED	NONE	NONE	Nugent et al., 2006
Beta. vulgaris	aadA	Nt Prrn1/ RBS	na	GFP	na	Nt psbA	De Marchis et al., 2008
D. carota	aadA	Nt PrrnF/RBS	Nt psbA	BADH	g10	Nt rps16	Kumar et al., 2004
G. hirsutum	aphA6	PrrnF/g10	Nt rps16	nptII	Nt psbA	Nt psbA	Kumar et al., 2004
Populus alba	aadA	Nt Prrn1/ RBS	Nt psbA	GFP	Nt psbA	Nt rps16	Okumura et al., 2006
Petunia hybrida	aadA	Nt Prrn1/Nt rbcL RBS	Nt psbA	GUS	*B. napus* Prrn	*B. napus* psbC	Zubko et al., 2004

Nt PrrnF: *N. tabacum* full length ribosomal operon promoter
RBS: plastid ribosome binding site GGAGG
Nt Prrn1: *N. tabacum* ribosomal operon promoter derivative including P1 PEP recognition specificity
Nt rbcL RBS: synthetic leader sequence derived from N. tabacum I RBS plus 18 bp leader Nt *rbcL* UTR:
g10: translation control region bacteriophage T7 gene 10
E. coli trc; rrnB: trc promoter contains the -35 region of the trp promoter with the -10 region of the lac promoter; transcriptional terminator of ribosomal operon B
Brs: *Brassica*
na: gene of interest expressed as dicistron with selectable marker

Methods and Materials

Vector construction

The pUC-based *L. sativa* long flanking plasmid (pLS-LF) was used to integrate foreign genes into the intergenic spacer between tRNA-Ile and tRNA-Ala genes of the plastid genome inverted repeat region (Ruhlman et al., 2007). A transformation cassette for the generation of transplastomic *L. sativa* plants that express CTB-Pins from the T7 gene 10 translational control region was transferred to pLS-LF from pZERO (Invitrogen, Carlsbad, CA). The cassette included the following published *N. tabacum* plastid regulatory sequence elements: ribosomal operon promoter (P*rrn*), *rps*16 and *psbA* 3' UTRs. The *aadA* (aminoglycoside 3-adenyl transferase) gene is included to confer spectinomycin resistance and is expressed via a GGAGG ribosome binding site. All digest products (vectors and inserts) were separated by electrophoresis through 0.8 % agarose-TAE (400 mM Tris-acetate, 10 mM EDTA) gels containing 1ug mL^{-1} ethidium bromide. Gel fragments were isolated using a sterile blade under UV illumination and DNA was eluted from gel fragments using the QIAquick gel extraction kit (Qiagen, Valencia, CA). Plasmid products of T4 ligase mediated reactions were transformed into *Escherichia coli* XL10-Gold® (Stratagene, La Jolla, CA) according to standard protocols (Sambrook et al., 1989). The expression cassette was digested with SnaBI. pLS-LF was digested with PvuII and treated with alkaline phosphatase prior to ligation with the SnaB1 digested cassette. Recovered plasmids were digested with SacI to determine correct orientation of the inserted cassette in pLS-LF. A transformation

cassette for the generation of transplastomic *L. sativa* plants that express CTB-Pins from *N. tabacum psbA* 5' and 3' UTR was assembled by digestion of the pLD-CtV-5CP plasmid (Ruhlman et al., 2007) with SalI and XbaI to release the CTB-Pins coding region plus the *N. tabacum psbA* 5' UTR. This fragment was ligated into a pUC intermediate plasmid upstream of the *N. tabacum psbA* 3' UTR. Nucleotide sequence of the intermediate plasmid called pUC-NtUTR-CTB-Pins was confirmed. This cassette was released by digestion with SalI and SnaBI and ligated into the modified pLS-LF (this vector now includes *aadA* gene with *L. sativa* native ribosomal operon promoter and 3' regulatory element for *rbcL*) which had been digested by SalI and EcoRV. All cloning steps were carried out in *E. coli* according to the methods of Sambrook et al. (1989). *Nicotiana tabacum* plants included in analyses are reported in Ruhlman et al. (2007). Transplastomic *L. sativa* plants expressing CTB-Pins from endogenous *psbA* 5' and 3' UTR were contributed by D. Burberry. Abbreviations for transplastomic lines in the following analyses are given in table 2.

Table 2: Transplastomic lines.

Nt-Nt-CP	*N. tabacum* background with endogenous *psbA* 5' and 3' UTR
Ls-Ls-CP	*L. sativa* background with endogenous *psbA* 5' and 3' UTR
Ls-Nt-CP	*L. sativa* background with *N. tabacum psbA* 5' and 3' UTR
Ls-g10-CP	*L. sativa* background with *N. tabacum* ribosomal operon promoter and T7 gene 10 translational control element

Seeds of *L. sativa* cv. Simpson elite (New England Seed Company, Hartford, CT) were surface sterilized in a 3% hypochlorite solution, rinsed three times in water and plated on Murashige and Skoog (Caisson, N. Logan, UT) media with 3% sucrose (MSO) and solidified with 5.8 g L^{-1} Phytablend® (Caisson). Young, fully expanded leaves ~4

cm^2 were placed adaxial side up on antibiotic free *L. sativa* regeneration (LR) media (Kanamoto et al., 2006). Leaves were bombarded with 0.6 µm gold particles (Bio-Rad, Hercules, CA) coated with pLS-LF-CTB-Pins (g10 or Nt-UTR) using the Bio-Rad PDS-1000/He Biolistic® device employing 900 psi rupture disks and a target distance of 6 cm as described by Kumar and Daniell (2004). Bombarded leaf samples were held in dark at 25 ^oC for two days prior to explant of 0.5 cm^2 pieces, adaxial side down onto LR media with 50 µg mL^{-1} spectinomycin dihydrochloride (Sigma Chemical, St. Louis, MO). Primary regenerants were screened by polymerase chain reaction (PCR) for the transplastomic event and positive shoots were subjected to an additional regeneration cycle on spectinomycin LR media. Following the second regeneration shoots were rooted in half strength MSO media with 50 µg mL^{-1} spectinomycin. Plants were propagated by rooting of nodal sections in half strength, hormone free MSO with spectinomycin. Rooted cuttings were hardened in Jiffy-7® peat pellets (Jiffy Products, Shippagan, N.B., CAN) before transfer to the greenhouse for seed production. Seeds (achene) were harvested when pappus was present, allowed to dry at 24 ^oC. Sterile seeds (100) collected from T0 transplastomic lines were plated on MSO media with 50 µg mL^{-1} spectinomycin along with wild type *L. sativa* to confirm maternal inheritance of plastid transgenes.

PCR screening of primary regenerants

To screen for integration of transgenes in the plastid genome ~100 ng genomic DNA was analyzed by PCR using primers 16SF (5'CAGCAGCCGCGGTAATACA

GAGGA3') and 3M (5'CCGCGTTGTTTCATCAAGCCTTACG-3'). Reactions were assembled in 50 µL total volume and performed in a PTC-100 Peltier thermal cycler (Bio-Rad), 5 µL of 10X PCR reaction buffer, 0.2 µM each dNTP, 0.5 µM 16SF and 3M primers, 1 µL of Mango Taq DNA polymerase (Bioline, Randolph, MA) and sterile distilled water. Template DNA was denatured at 94 °C for 5 min and 30 cycles of amplification was carried out as follows: 94°C for 1 min 60°C for 30 sec (annealing), and 72°C for 2 min (extension) and 94 ^{o}C for 1 min (denaturation). Final extension was performed at 72 °C for 10 min. PCR products were examined by electrophoresis of 5 µL aliquots through 0.8 % agarose-TAE gels containing 1 µg mL^{-1} ethidium bromide under UV illumination.

Southern blotting confirmation of homoplasmy

Isolation of genomic DNA was performed on young, *in vitro* grown leaves. Samples were ground in liquid nitrogen with chilled, sterile mortar and pestle. Each sample, 75 mg, was transferred to a 2 mL microfuge tube and extraction was carried out using a QIAGEN DNeasy® Plant mini kit (#69104) according to the manufacturer's protocol. Each column was eluted with 200 µL sterile nuclease-free water and quantified by spectrophotometry. Five micrograms of total DNA was digested to completion with BglII (Ls-g10-CP) or AflIII (Ls-Nt-CP lines), separated on 0.8% TAE-agarose gels and transferred to Nytran SPC nylon membranes (Whatman Inc., Sanford, ME) by capillary action. Plastid flanking sequence probe (1.3 kb) was amplified by PCR from *L. sativa* genomic DNA. PCR product was column purified and labeled probe was

generated by incubation with α-^{32}P-dCTP and Ready-To-Go™ DNA Labeling Beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blots were pre-hybridized for one hour at 68 ^{o}C in QuikHyb® reagent (Stratagene). Hybridization was initiated with addition of labeled probe (1.25 x 10^6 cpm mL^{-1}) and sonicated salmon sperm DNA at 68 ^{o}C with and continued for one hour. Blots were washed twice at 37 ^{o}C in 2X SSC (0.3 M sodium chloride, 30 mM sodium citrate, pH 7) and twice at 65 ^{o}C in 0.1X SSC. Radiolabeled blots were exposed to film on intensifying screens at -80 ^{o}C for 16 hours.

Polyribosome association assay and northern blotting

Green leaves from *in vitro* grown plants were ground to a fine powder in liquid nitrogen. Approximately 300 mg of each sample was transferred to a 2 mL microcentrifuge tube and vortexed with one mL extraction buffer (0.2 M Tris HCl pH 9, 0.2 M KCl, 35 mM MgCl2, 25 mM EGTA pH 8.3, 1% Triton X100, 2% polyoxyethylene-10-tridecylether) supplemented with 0.5 mg mL^{-1} heparin, 100 µg mL^{-1} chloramphicol (Sigma), 25 µg mL^{-1} cyclohexamide (Sigma). Homogenates were forced gently through glass wool packed in 3 mL syringe into microcentrifuge tube on ice and extracts were held on ice for 10 min. Extracts were centrifuged at 17, 900 x *g* for 5 min at 4 ^{o}C. Supernatants were transferred to new tubes and 1/20th volume of 10% sodium deoxycholate (Sigma) was added. Control reactions were incubated with puromycin (3 mg mL^{-1}; Sigma) at 37 ^{o}C for 10 min. All reactions were held on ice for 5 min prior to centrifugation at 17, 900 x *g* for 15 min at 4 ^{o}C. Supernatants (500 µL) were layered onto 15%-30%-40%-50% sucrose gradients in 10X salts (0.4 M Tris-HCl, pH 8, 0.2 M

KCl, 0.1 M $MgCl_2$) prepared in Beckman Ultra-Clear™ centrifuge tubes (13 x 51 mm; #344057, Beckman Coulter, Fullerton, CA). One aliquot of supernatant from each sample was reserved for isolation of total RNA. Gradients were centrifuged at 4 ^{o}C in SW55-ti rotor for 65 min at 192, 000 x *g*. Fractions of approximately 500 µL were collected into microfuge tubes containing 50 µL of 5% SDS and 0.2 M EDTA pH 8, by puncturing gradient tubes with an 18 gage needle. One volume of phenol:chloroform:isoamyl alcohol (25:24:1; Sigma) was added to each fraction and vortexed then centrifuged at 17, 900 x *g* for 5 min. The aqueous phase was transferred to a new tube, two volumes of absolute ethanol was added and mixed by inversion. RNA was pelleted by centrifugation, supernatant was discarded and pellets were dried under vacuum. Pellets were resuspended in 30 µL Tris-EDTA. RNA sample buffer (80 mM MOPS, pH 7, 4mM EDTA, 0.9 M formaldehyde, 20% glycerol, 30.1% formamide, 5 mM sodium acetate, 0.25% bromophenol blue) was added and fractions were separated by electrophoresis under denaturing conditions (gel: 20 mM MOPS, pH 7, 5 mM sodium acetate, 1.2 % agarose, 1 mM EDTA, 2.5 M formaldehyde, 0.1 µg mL^{-1} ethidium bromide; buffer: 20 mM MOPS, pH 7, 5 mM sodium acetate, 1 mM EDTA) for 90 min at 70 volts. Ribosomal RNAs were visualized by UV-imager (Bio-Rad GelDoc 2000). Gels were washed twice for 15 m in RNase-free water and RNA was transferred to Nytran SPC nylon membranes (Whatman) by capillary action in 20X SSC. Blotted membranes were rinsed in RNase-free water and fixed by UV cross linking. The full length CTB-Pins coding region was used to generate α-^{32}P-labeled, single stranded DNA probes according to the procedure described above for Southern blotting. Pre-

hybridization and hybridization steps for polyribosome blots were carried out in Denhardt's buffer (0.1% ficoll, 0.1 % polyvinylpyrolidone, 0.1% bovine serum albumin, 5X SSC, 20 mM sodium phosphate, 7% SDS). For analysis of total transcripts, RNA was extracted using the Qiagen RNeasy®Mini Kit, and quantified by spectrophotometry. Total RNA, 2.5 µg, was electrophoresed and blotted as described above.

Western blot and densitometric analysis

Second generation (T_1) CTB-Pins transplastomic *N. tabacum* and *L. sativa* were raised in the UCF greenhouse. Young, mature and older, fully expanded leaves from ~8 week old plants were harvested in August at 5:00 a.m., 10:00 a.m., 2:00 p.m. and 6:00 p.m., ground in liquid nitrogen and stored at -80 ^{o}C. Approximately 100 mg of leaf tissue was suspended in five volumes of protein extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl pH 8, 0.1% Triton X-100, 100 mM DTT, 400 mM sucrose, 2 mM PMSF). Extractions were vortexed vigorously for 20 min at 4 ^{o}C prior to determination of total protein using Bio-Rad Protein Assay Reagent (Bio-Rad). Total leaf proteins along with 100, 200, 400 and 600 ng of purified bacterial CTB (Sigma) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Trans-Blot® nitrocellulose membranes (Bio-Rad) for immunoblotting, according to Kumar and Daniell (2004). Membranes were rinsed in sterile water and blocked in PBS with 3% skim milk and 0.1% Tween 20 (Sigma).

Immunoblotting with anti-cholera toxin primary antibody (1:3500, Sigma) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:4000,

Southern Biotech, Birmingham, AL) was employed for spot densitometric analysis of western blots. A SuperSignal® West Pico HRP Substrate Kit (Pierce, Rockford, IL) was used for detection of chemiluminescence signal by exposure to film (Molecular Technologies, St. Louis, MO). Films were analyzed using AlphaImager® and AlphaEase® FC software (Alpha Innotech, San Leandro, CA). Tissue collected at the latest developmental stage was prepared as above and subjected to SDS-PAGE along with CTB standards ranging in concentration from 0.5 to 3 µg. Gels were stained with Coomassie blue and used for densitometric quantitation.

Nicotiana tabacum plastid isolation

Intact *N. tabacum* plastids were isolated according to Yukawa et al. (2007). Fully expanded green leaves (200 g) were collected from 4-5 week old greenhouse grown *N. tabacum* cv TN90. Leaves were rinsed in cold water and homogenized in 50 g batches with 150 mL MCB1 (50 mM HEPES/KOH pH 8.0, 0.3 M mannitol, 2 mM EDTA, 5 mM BME, 0.1% BSA 0.6 % PVP). Homogenates were filtered through four layers of cheesecloth then two layers of Miracloth (Calbiochem) and centrifuged for 5 min at 1, 000 x *g* in a Sorvall SS-34 fixed angle rotor at 4 ^{o}C. Pellets were resuspended by gentle agitation with a soft paintbrush in 30 mL MCB1 and 5 mL was layered onto 25 mL Percoll (Sigma) gradients (20%-50%-80% in MCB1). Gradients were centrifuged for 10 min at 10, 000 x *g* in L-90K ultracentrifuge SW32-ti rotor; 2 ^{O}C. The lower dark green band containing intact plastids was harvested, washed in three volumes MCB2 (50 mM HEPES/KOH pH 8.0, 0.32 M mannitol, 2 mM EDTA, 5 mM BME). Plastids

were collected by one min centrifugation at 600 x *g* in Sorvall SS-34 at 4 ^{o}C. Plastid pellet was resuspended in minimal volume of EMSA binding buffer (20 mM Tris–HCl, pH 8.5, 20 mM KCl, 10 mM MgCl2, 5 mM DTT, 2 mg ml^{-1} heparin) with rigorous agitation to disrupt plastid envelopes. Membranes were sedimented by centrifugation in SS-34 rotor; 27, 000 x *g* for 15 m. Supernatants (stromal extracts) were adjusted to 15% glycerol, aliquoted and stored at -80 ^{O}C.

***Lactuca sativa* plastid isolation**

Intact *L. sativa* plastids were isolated according to Gruissem et al. (1986). Fully expanded green leaves (200 g) of 6-8 week old hydroponically grown *L. sativa* cv longifolia were collected from the greenhouse and washed in cold water. Leaves were homogenized in 50 g batches with 150 mL 1X GM buffer (50 mM HEPES/KOH pH 6.8, 1 mM sodium pyrophosphate' 33 mM sorbitol, 2 mM EDTA, 1.25 mM magnesium chloride, 1.25 mM manganese chloride, 2 mM dithiolthreitol). Homogenates were filtered through four layers of cheesecloth then two layers of Miracloth and centrifuged for 5 min at 1, 000 x *g* in a Sorvall SS-34 fixed angle rotor at 4 ^{o}C. Pellets were resuspended by gentle agitation with a soft paintbrush in 30 mL 1X GM buffer and 5 mL was layered onto 25 mL PCBF (100 mL Percoll, 3% polyethylene glycol 6000, 1% bovine serum albumin, 1% ficoll) density gradients (10%: 6 mL 5X GM, 3 mL PCBF, 1 mM glutathione, 21 mL water; 80%: 6 mL 5X GM, 24 mL PCBF, 1 mM glutathione). Gradients were centrifuged for 20 min at 8, 100 x *g* in L-90K ultracentrifuge SW32-ti rotor; 2 ^{O}C. The lower dark green band containing intact plastids was harvested,

washed in two volumes 1X GM buffer (50 mM HEPES/KOH pH 8.0, 0.32 M mannitol, 2 mM EDTA, 5 mM BME). Plastids were collected by 3 min centrifugation at 1, 500 x *g* in Sorvall SS-34 at 4 ^{o}C. Plastid pellet was resuspended in minimal volume of EMSA binding buffer with rigorous agitation to disrupt plastid envelopes. Membranes were sedimented by centrifugation in SS-34 rotor; 27, 000 x *g* for 15 min. Supernatants (stromal extracts) were adjusted to 15% glycerol aliquoted and stored at -80 ^{O}C.

***In vitro* transcription of *psbA* 5' UTR and Ls-UTR-CTB mRNA**

Plasmids (pBluescript SK+, Stratagene) containing the *psbA* 5' UTR were digested with NdeI (*L. sativa*) *or* NcoI (*N. tabacum*) to generate linearized templates for T7 *in vitro* transcription using the MAXIscript® Kit (Ambion Inc, Austin TX) according to manufacturer's instructions. For mRNA turnover the *L. sativa* UTR-CTB-Pins plasmid was digested at the XbaI site situated at the 3' end of CTB-Pins. For labeled UTR species uridine triphosphate (UTP) was replaced with 3.125 μM α-^{32}P UTP (Perkin Elmer, Waltham, MA). Reaction products were separated by denaturing polyacrylamide and eluted following manufacturer's instructions. Following ethanol precipitation supernatants were discarded and RNA pellets were vacuum dried. Pellets were resuspended 50 μL nuclease-free water and quantified by spectrophotometry and liquid scintillation counting. Single use aliquots were prepared and stored at -80 ^{o}C.

Electrophoretic Mobility Shift and mRNA turnover

Competitive RNA EMSA was adapted from Alexander et al. (1998). Stromal extracts were thawed on ice and total protein content was determined using the Bio-Rad Reagent (Bio-Rad). Stromal proteins of *N. tabacum* (20 µg) or *L. sativa* (40 µg) were incubated with 5 fmoles of endogenous radiolabeled *psbA* 5'UTR, with or without unlabeled competitor *psbA* 5' UTR. All reactions were supplemented with 0.5 µg uL^{-1} yeast tRNA (Ambion) to reduce non-specific binding and total volume was adjusted to 20 µL using EMSA binding assay buffer. Control reactions included no competition, competition with 50X molar excess of unlabeled native UTR and labeled probe only (no protein). Experimental reactions contained 50X, 100X and 200X molar excess of unlabeled non-native competitor UTR (i.e. *N. tabacum* protein with *L. sativa* UTR as competitor). Competitors were added 5 min prior to the labeled probe. Reactions were allowed to proceed for 15 min at 22 ^{o}C in the presence of labeled probe. 4 µL of 5X non-denaturing gel loading buffer was added (225 mM Tris-HCl, pH 6.8, 50% glycerol, 0.05% bromophenol blue) and reactions were separated through 8% polyacrylamide.

For degradation analysis of the labeled Ls-5'UTR-CTB-Pins transcript in stromal extracts the no competition control reaction described above was used. At the designated time points total RNA was isolated by phenol:chloroform as described below and extracts were separated by electrophoresis. Gels were vacuum dried and exposed to film for 6-12 hrs for analysis of binding reactions.

Pulse-chase labeling and immunopreciptation

Fully expanded leaves of *L. sativa* and *N. tabacum* transplastomic lines were harvested under different growth conditions as described in results. Leaves were cut into 5 mm^2 explants under half strength MSO media with a sharp, sterile blade. Explants, approximately 50 per sample were placed in a vacuum apparatus containing 10 mL MSO media with 0.05 % Tween 20 and 1 mCi EXPRESS ^{35}S protein labeling mix (PerkinElmer). Explants were infiltrated for 90 sec, transferred to a Petri dish along with the media and incubated in the light for one hour. Explants were removed from isotope media, rinsed with sterile water and transferred to the vacuum apparatus with fresh media without isotope containing 10 mM methionine. Infiltration was repeated and explants and media were transferred to a Petri dish for duration of chase period under 16 hours light and 8 hours dark in the growth chamber. At the time intervals shown in results, explants (5-6 each; ~25 mg) were removed from the media, blotted on tissue paper and transferred to microcentrifuge tubes. Samples were frozen in liquid nitrogen and stored at -80 ^{o}C until the end of the chase period.

Samples were ground to a fine powder using a glass pestle with liquid nitrogen, in the microcentrifuge tubes. Approximately eight volumes of homogenization buffer (200 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM EDTA, 0.1% SDS , 0.05% Tween 20, 2 mM PMSF) was added to each sample followed by vortexing with a micropestle. Total protein in homogenates was quantified using the Bio-Rad Protein Assay Reagent (Bio-Rad). An equal amount of total protein was taken from each sample and diluted to one mL in phosphate buffered saline (PBS; 100 mM, KCl 27 mM, NaCl 1.37M, pH 7.4)..

Primary antibody (α-cholera toxin from rabbit, 1:500, Sigma) was added to each sample and tubes were placed on a rocker at 4 ^{o}C for 4-6 hours. To each tube 40 µL protein A-agarose (Santa Cruz Biotech, Santa Cruz, CA) was added and samples were incubated on the rocker at 4 ^{o}C for 16 hours. Samples were pelleted by pulse centrifugation and aspirated. Pellets were washed three times with 500 µL PBS. Final pellet was suspended in 50 µL electrophoresis sample buffer (90 mM Tris HCl pH 6.8, 20% glycerol, 2% SDS, 0.02% Bromophenol blue, 100 mM dithiolthreitol) and boiled for two min. Samples were centrifuged and half of the supernatant was used for scintillation counting, while the other half was analyzed by SDS-PAGE. SDS gels were dried and exposed to film to visualize immunoprecipitated, labeled protein.

Genomic analyses

The complete plastid genome of *D. carota* (Ruhlman et al, 2006) was analyzed for size, gene content and order and repeat structure using Dual Oraganellar GenoMe Annotator (DOGMA; Wyman et al., 2004) and Comparative Repeat Analysis (CRA). The settings for identifying direct and inverted (palindromic) repeats in CRA included a size range between 30-5, 000 base pairs (bp) and a Hamming distance of 3 (limiting hits to sequence identity of ≥ 90%). The annotated sequence was exported from DOGMA as a text file in GenVision (DNASTAR, Inc, Madison WI) format. This text file was manipulated in Microsoft® Excel (Microsoft, Bellvue, WA) to orient the genes on appropriate stands and demarcate the inverted repeats, large and small single copy regions on the circular genome map creating five separate Excel worksheets that were

converted to text files for import to GenVision. The GenVision plug in interfaces with Adobe® Illustrator® (Adobe Systems, San Jose, CA) allowing partial automation of map drawing provided the data files imported from Excel contain the correct sequence information.

Sequences of intergenic spacer regions upstream from genes representing different functional groups (Table 3) were extracted from complete plastid genomes on GenBank for 20 species representing most major clades of angiosperms, including four grasses and three legumes (Table 4). Alignments were anchored by the inclusion of 100 bases from the coding region of adjacent genes. Sequences were aligned using MUSCLE (Edgar, 2004), followed by manual adjustment in Geneious (Drummond et al., 2008), and sequence identity was calculated for the region encompassed by 200 bases upstream of the translation start codon, i.e. promoters and UTRs.

Table 3: Intergenic regions include in analysis

rbcL - atpB	*ycf3 - psaA*
psbB - clpP	*trnK* (UUU) - *psbA*
*rbcL - accD**	*rpoC2-rps2*
trnC (GCA) - *rpoB***	*cemA - petA*
trnV (GAC) - *rrn16*	*ndhF - rpl32*
psbE - petL	

**accD* is missing in *Sorghum, Hordeum, Oryza, and Zea* ; in *Glycine, Medicago, and Cicer*: *accD-rps16*

***Helianthus and Lactuca*: *rpoB- trnE*-UUC

For reference and comparison the sequence identity was also determined for the aligned coding regions. Geneious was used to conduct motif searches for the

identification of functional domains such as promoter elements and *cis* translation factors.

Table 4: Taxa included in genomic analyses.

Family	Genus and species	NCBI accession number
Fabaceae	*Glycine max*	NC_007942
Fabaceae	*Medicago truncatula*	NC_003119
Fabaceae	*Cicer arietinum*	NC_011163
Euphorbiaceae	*Manihot esculenta*	NC_010433
Asteraceae	*Lactuca sativa*	NC_007578
Asteraceae	*Helianthus annuus*	NC_007977
Apiaceae	*Daucus carota*	NC_008325
Solanaceae	*Nicotiana tabacum*	NC_001879
Solanaceae	*Solanum lycopersicum*	DQ347959
Rubiaceae	*Coffea arabica*	NC_008535
Vitaceae	*Vitis vinifera*	NC_007957
Poaceae	*Sorghum bicolor*	NC_008602
Poaceae	*Hordeum vulgare*	NC_008590
Poaceae	*Oryza sativa*	NC_001320
Poaceae	*Zea mays*	NC_001666
Malvaceae	*Gossypium hirsutum*	NC_007944
Amaranthaceae	*Spinacia oleracea*	NC_002202
Brassicaceae	*Brassica rapa*	DQ231548
Rutaceae	*Citrus sinensis*	NC_008334
Cucurbitaceae	*Cucumis sativus*	NC_007144

Results

Vector construction:

The pUC-based *L. sativa* long flanking plasmid (pLS-LF) was used to integrate foreign genes into the intergenic spacer between tRNA-Ile and tRNA-Ala genes of the plastid genome (Ruhlman et al., 2007). The pLS-LF vector has been modified to include the *aadA* gene for selection of transplastomic lines (Figure 1A). Expression of *aadA* is driven by the *L. sativa* endogenous P*rrn* and a GGAGG ribosome binding site.

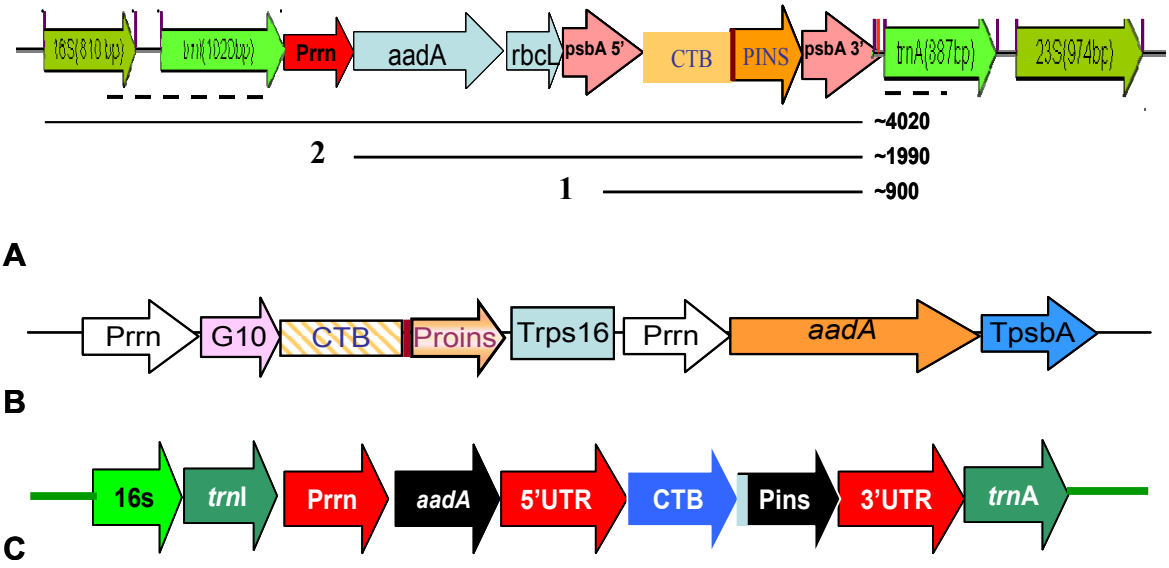


Figure 1: Schematic representation of expression cassettes:
Schematic representation of insertion region and transplastomic mRNA. A) Mono- and dicistron (~900[1] and 1900[2] bases) are transcribed from inserted promoters; longer transcripts result from the endogenous promoter for ribosomal operon. *psbA* 5' and 3' UTR are derived from *N. tabacum* or *L. sativa*, Dashed line represents hybridization region for Southern using the 1.6 kb *L. sativa* flanking probe. B) g10 integration cassette for *L. sativa* and C) integration site and cassette for *N. tabacum* expression from native UTR.

The *aadA* transcript is stabilized by inclusion of the endogenous 3' UTR of *rbcL* (Verma et al., 2008). The pLS-LF-Ls-Nt-CP plasmid was constructed by transferring the *N. tabacum* expression cassette for Nt*psbA*-CTB-Pins-Nt*psbA* from pLD-5CP-13 (Figure 1C; Ruhlman et al., 2007) to the modified pLS-LF backbone.

Generation of transplastomic lines and confirmation of homoplasmy

Plastid transformation of *L. sativa* and *N. tabacum* was carried out as described in methods section. Primary regenerants were identified by PCR and subjected to an additional round of regeneration followed by rooting in selective media

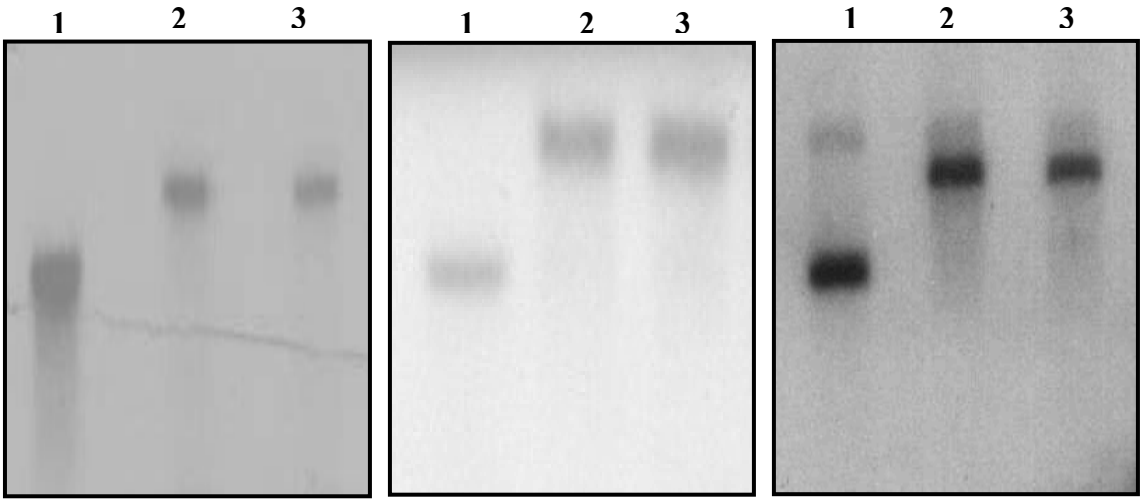


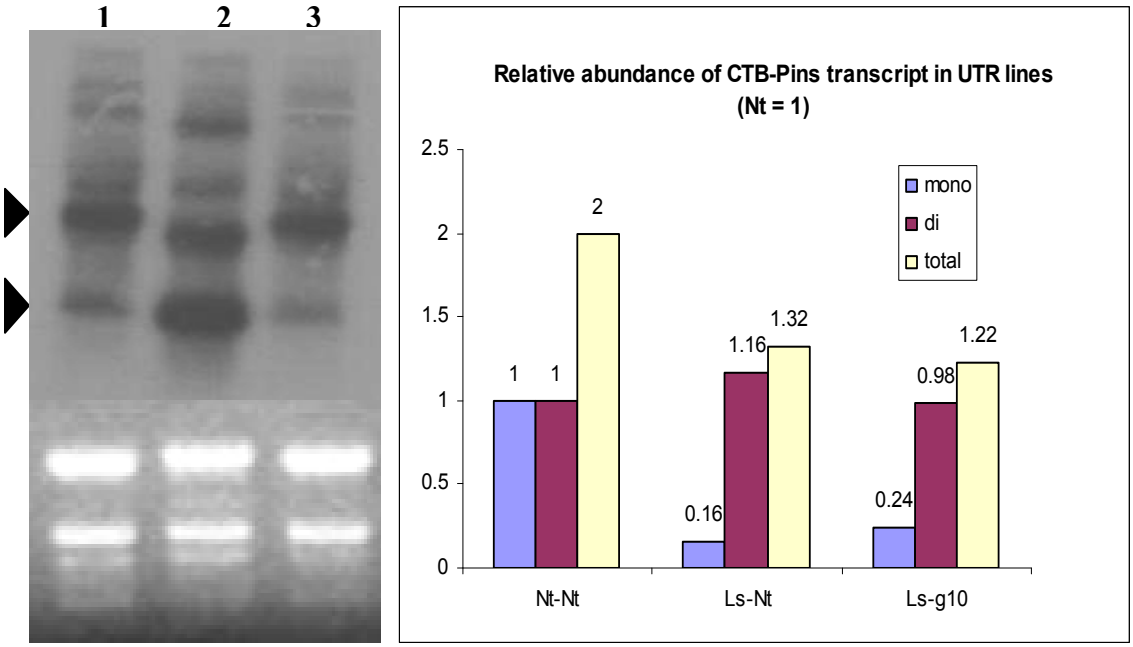
Figure 2: Confirmation of homoplasmy by Southern hybridization.
Southern blots of AflIII- digested genomic DNA from T_0 (A), T1 (B) Ls-Nt-CP and C) Ls-g10-CP T_1 lines were probed with radiolabeled *L. sativa* flanking sequence probe. Lanes 1) wild type, 2 & 3) two independent Ls-Nt-CP lines.

Site specific integration and homoplasmy of the transplastome was confirmed by Southern hybridization with flanking sequence probes specific for *L. sativa* (Figure 2) or *N. tabacum.* Generation and confirmation of homoplasmy for CTB-Pins line Nt-Nt-CP has been reported previously (5CP-13; Ruhlman et al., 2007). All transplastomic lines

were found to be homoplasmic, containing no detectable wild type copies of the plastome.

Foreign gene transcripts vary in size and abundance with endogenous or heterologous expression elements.

Total RNA was isolated from different transplastomic lines and northern blots were prepared to examine the transcript populations generated from various regulatory elements. Blots were probed with radiolabeled coding regions for the gene of interest (Figure 3A).



A **B**

Figure 3: Foreign gene transcripts vary in size and abundance with endogenous or heterologous expression elements.
Total RNA (2.5 µg) was separated by electrophoresis, blotted to nylon membranes and probed with radiolabeled CTB-Pins. A) Upper frame, autoradiograph; lower frame; ethidium bromide stained rRNA. Lane 1, Ls-g10-CP; lane 2) Nt-Nt-CP; lane 3) Ls-Nt-CP. Arrows indicate mono- and dicistronic transcripts for CTB-Pins; see Figure 1A for sizes B) Desitometric quantitification for relative abundance of transcripts represented relative to Nt-Nt-CP line.

Relative abundance of CTB-Pins transcript species was assessed by densitometry and varied between the different transplastomic lines (Figure 3B). All of the plants under analysis carry two engineered promoters, one upstream of *aadA* and one upstream of the gene of interest, allowing for transcription of monocistrons, and also dicistrons arising from RNA polymerase read through of the upstream 3' UTR element where present. While accumulation of dicistronic mRNA for CTB-Pins varied less dramatically, *N. tabacum* plants with endogenous *psbA* UTR expressed 84% and 76% more monocistronic mRNA and 66% and 61% more total mRNA for CTB-Pins than *L. sativa plants* with the *N. tabacum* element or the g10 element, respectively. As seen in transplastomic lines where genes are integrated within the ribosomal operon, a number of larger species were detectable using the CTB-Pins probe but were not quantified for this analysis.

Foreign gene transcripts that include endogenous *psbA* 5' UTR were stabilized in non-polysomal fractions isolated from transplastomic *L. sativa.*

Total RNA was prepared from fractions separated through sucrose gradients to evaluate polysome association of foreign gene transcripts in *L. sativa* transformants that express CTB-Pins from the endogenous *psbA* UTR (Ls-Ls-CP) versus the *N. tabacum psbA* 5' UTR (Ls-Nt-CP). The distribution of ribosomal RNAs in gradient fractions was similar between the two lines. Northern blots of 12 fractions collected from the bottom of the gradient were probed with the radiolabeled, full-length coding sequence for CTB-Pins to localize transplastomic transcripts. Blots prepared from all fractions off the

gradient for Ls-Ls-CP or LS-Nt-CP lines show that the CTB-Pins transcript present in all fractions was predominantly monocistronic, although the dicistron is readily detectable and in some fractions abundant (Figure 4A & B).

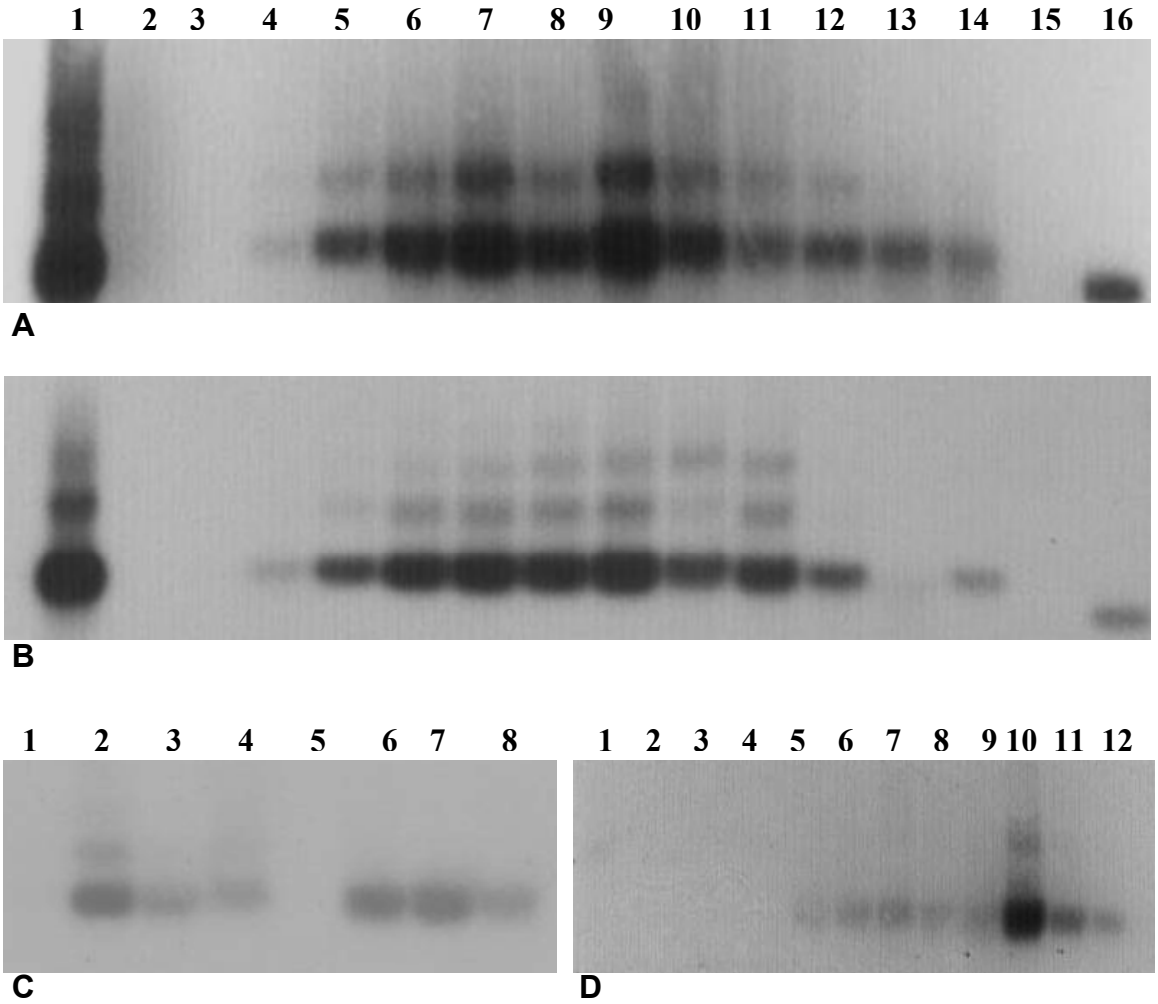


Figure 4: Polysome-free CTB- Pins transcripts are stabilized in lines with the endogenous *psbA* 5' UTR.

A) Sucrose gradient fractions were separated through 1.2% agarose and transferred for northern blotting with CTP-Pins probe. Lanes are numbered above (A) for both A (Ls-Ls-CP) and B (Ls-Nt-CP). Lane 1) total RNA, 2) blank, 3-14) fractions 1-12 collected from the bottom of the gradient, 15) RNA standards 16) CTB-Pins probe. C) Lanes 1-4 and 5-8) fractions 1, 4, 8, 11 from Ls-Nt-CP and Ls-Ls-CP, respectively. D) Controls; lanes 1-3) pooled fractions from wt sample, 4) blank. Lanes 5-7) each lane contains 2 pooled factions from 2-7 of puromycin-treated sample (Ls-Ls-CP). In lanes 8-12 fraction corresponds to lane number.

One larger CTB-Pins mRNA species was observed only in Ls-Nt-CP lines, this likely corresponds to a processed intermediate originating from the endogenous P*rrn* (see Figure 1A for predicted transcript sizes). Foreign mRNA is more abundant in Ls-Ls-CP lines in the upper fractions suggesting that the transcript pool that is not associated with polysomes is stabilized in these lines (Figure 4A). To account for variation in transfer and hybridization efficiency that can confound blot to blot comparisons, selected fractions were prepared from an independent isolation of these two lines and were blotted together to confirm this observation (Figure 4C). Wild type samples and puromycin-treated controls are shown in figure 4D. Autoradiographs were used for densitometry. In Ls-Nt-CP line 22-37% of total signal was associated with the two non-polysomal fractions, whereas in the Ls-Ls-CP line 40-65% of the total signal was found in these fractions.

Foreign protein accumulation varied widely with the use of endogenous or heterologous regulatory elements.

Second generation (T_1) transplastomic plants of *L. sativa* and *N. tabacum* expressing CTB-Pins were grown in the greenhouse for 8-10 weeks. Leaves were harvested representing different developmental stages at four time points during the light cycle to evaluate foreign protein accumulation. Expression of CTB-Pins was quantified by densitometric analysis of crude homogenates against known quantities of CTB standard (Figure 5 & 6B). For these quantitative studies homogenates have been used as we have found that up to 90% of CTB-Pins protein is retained in the pelleted

fraction following centrifugation. We modified our protein extraction and SDS sample loading buffer to include 100 mM DTT, and increased the buffer to tissue ratio in extractions to enhance solubility of CTB-Pins. In all three lines analyzed, the oldest leaves showed the highest levels of CTB-Pins accumulation, up to 72% of total leaf protein (TLP) in *N. tabacum* lines, 12.3% of TLP in Ls-Nt-CP and ~25% of TLP in Ls-g10.

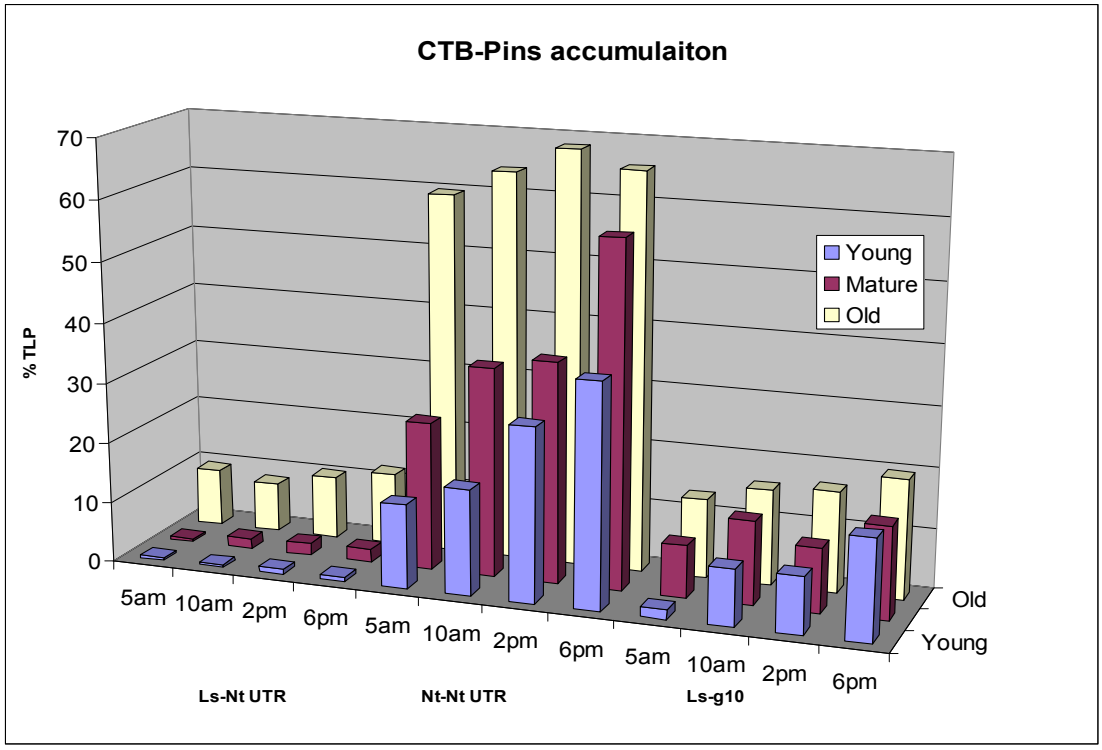
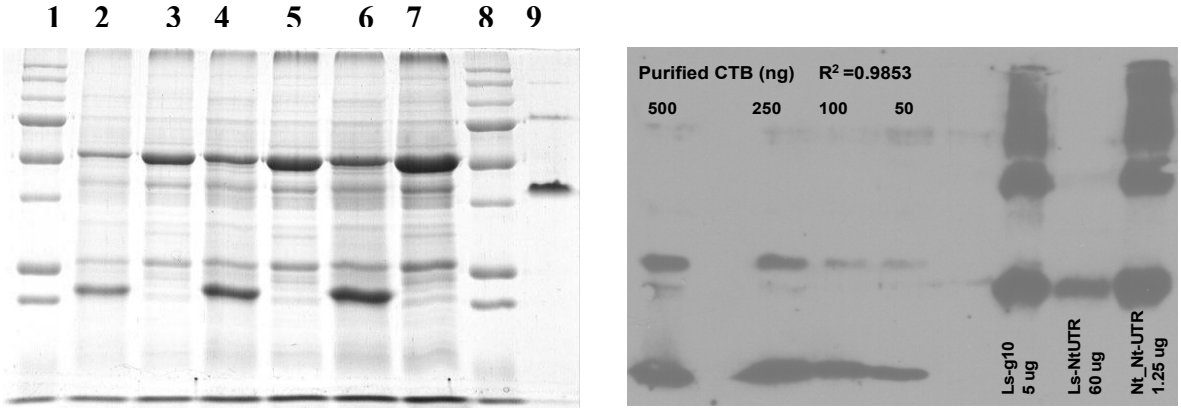


Figure 5: Accumulation of CTB-Pins in transplastomic *L. sativa* and *N. tabacum*. A) foreign protein accumulation estimated by densitometry using known quantities of CTB standard presented as a function of light and developmental stage. Means and standard deviations are presented in table 5.

To confirm this estimate for mature *N. tabacum* leaves we quantified samples by densitometric analysis of Coomassie stained gels (Figure 6A). We estimated that in these lines CTB-Pins accumulation exceeds that of Rubisco by a factor of up to two. In

mature leaves harvested at 6:00 p.m. the *N. tabacum* line reached 57% +/- 2.12, whereas the *L. sativa* line accumulated CTB-Pins to 2.15% +/- 0.76 of TLP, a 96 % reduction in foreign protein, despite the fact that both of these lines express CTB-Pins from the *N. tabacum psbA* 5' UTR. The expression pattern observed in Nt-Nt-CP young and mature leaves was consistent with developmental and light regulation with increasing levels of foreign protein as maturing leaves synthesize and accumulate protein throughout the light cycle.



A **B**

Figure 6: CTB-Pins accumulated to high levels in transplastomic lines.
A) Coomassie stained SDS-PAGE of transplastomic *N. tabacum*. Lanes 2, 4, 7: 10, 20 & 30µg TLP from older leaf at 2pm, respectively. Lanes 3, 5 & 7: equal protein from same stage wild type leaves; lane 9: 5 µg CTB. B) representative western blot probed with α-CTB.

Nominal differences in samples taken from various developmental stages and light conditions were observed in the two *L. sativa* lines and were attributed to the overall physiological condition of the plants and experimental variation. Means and standard deviation for all data represented in figure 5A are given in Table 5.

CTB-Pins is stable in transplastomic plants over time

To evaluate the stability of CTB-Pins in transplastomic lines we performed protein labeling with ^{35}S and extracted leaf proteins at different time points throughout the chase period. Immunoprecipitation with α-Cholera toxin antibody was used to isolate the CTB-Pins protein away from the other labeled proteins translated during the pulse period. Figure 7 shows result from scintillation counting of immunoprecipitates of one experiment where leaf samples were harvested from the greenhouse at 2:00 pm

Table 5: Yield of CTB-Pins in transplastomic lines.
Values are presented in percent of total leaf protein as means from at least three independent assays with standard deviations.

	Ls-Nt-CP					
	Young leaves		Mature leaves		Older leaves	
	Mean	SD	Mean	SD	Mean	SD
5:00 AM	0.46	0.07	0.51	0.01	9.53	0.68
10:00 AM	0.44	0.09	1.61	0.09	8.14	0.50
2:00 PM	0.85	0.14	2.00	0.72	10.37	2.89
6:00 PM	0.75	0.16	2.15	0.77	11.97	0.35
	Nt-Nt-CP					
	Young leaves		Mature leaves		Older leaves	
	Mean	SD	Mean	SD	Mean	SD
5:00 AM	13.95	3.75	24.55	5.02	59.70	11.60
10:00 AM	17.53	4.11	38.70	7.73	64.05	3.04
2:00 PM	28.73	2.69	36.40	4.27	68.37	5.45
6:00 PM	36.93	1.72	57.00	2.12	65.65	0.21
	Ls-g10-CP					
	Young leaves		Mature leaves		Older leaves	
	Mean	SD	Mean	SD	Mean	SD
5:00 AM	1.63	0.06	8.73	1.80	13.20	2.23
10:00 AM	9.43	1.65	13.85	2.62	15.85	0.92
2:00 PM	9.52	1.38	10.70	0.71	16.63	4.34
6:00 PM	16.67	1.10	15.39	4.93	19.77	4.53

As we found with samples harvested from other growth conditions, we were unable to visualize ^{35}S signal in SDS gels, despite long exposure to autoradiographic film and CPM (counts per minute) values for scintillation counting were low despite strong

labeling detected in the total homogenates prior to immunoprecipitation for the CTB moiety. While there was some fluctuation in the signal over time for all the lines examined, the immunoprecipitated protein, CTB-Pins, appeared stable over the 72 hour chase period.

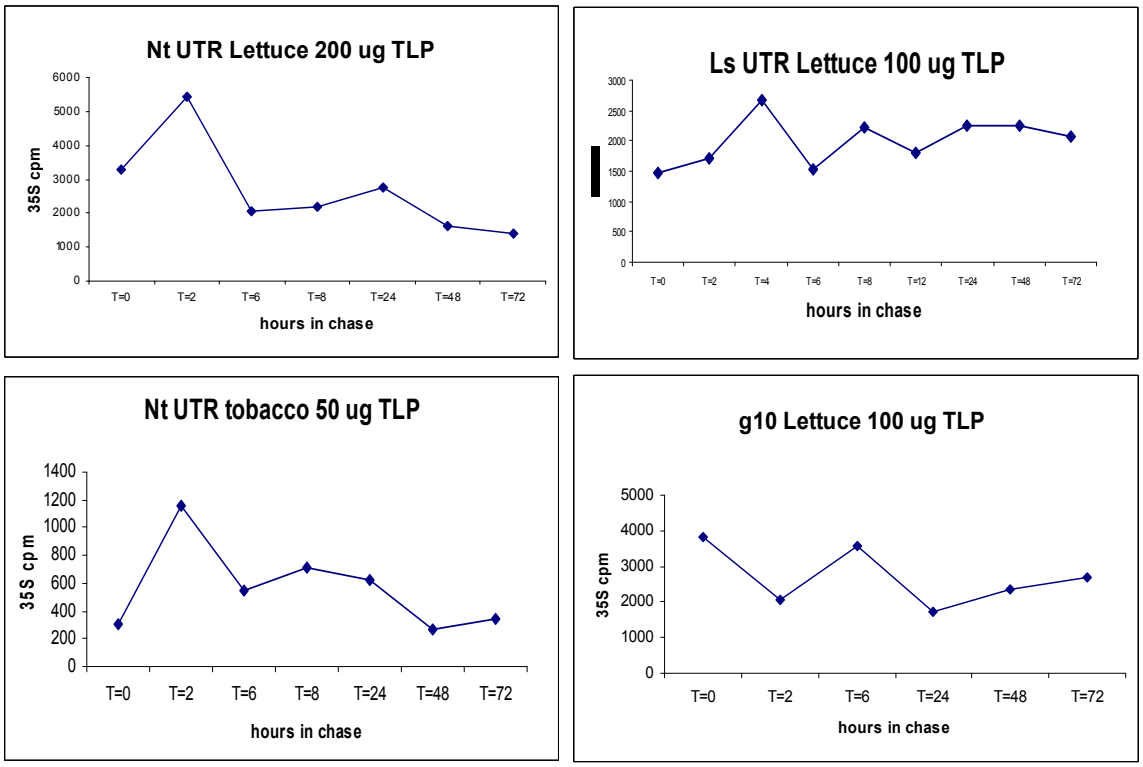


Figure 7: Stability of CTB-Pins was similar in all transplastomic lines.
Total translation products were labeled for one hour with 35S and samples were taken over a time course as shown on the X axis. Protein extracts were immunoprecipitated with α-Cholera toxin antibody and analyzed by scintillation counting.

Stromal RBPs preferentially associate with endogenous *psbA* 5' UTR providing transcript stability.

Extracts of plastid stromal proteins were prepared for *L. sativa* and *N. tabacum* as described in methods. Labeled, full length *psbA* 5' UTRs specific to each system were transcribed *in vitro* as were the unlabeled competitors. Reactions were

separated in native gels to evaluate the affinity of native UTRs for stromal protein binding in the presence of up to 200-fold molar excess of competitor species. As shown in figure 8, in the *L. sativa* (A) and *N. tabacum* (B) system, heterologous (unlabeled) *psbA* 5' UTR was an ineffective competitor for association with RBPs in the stromal extracts.

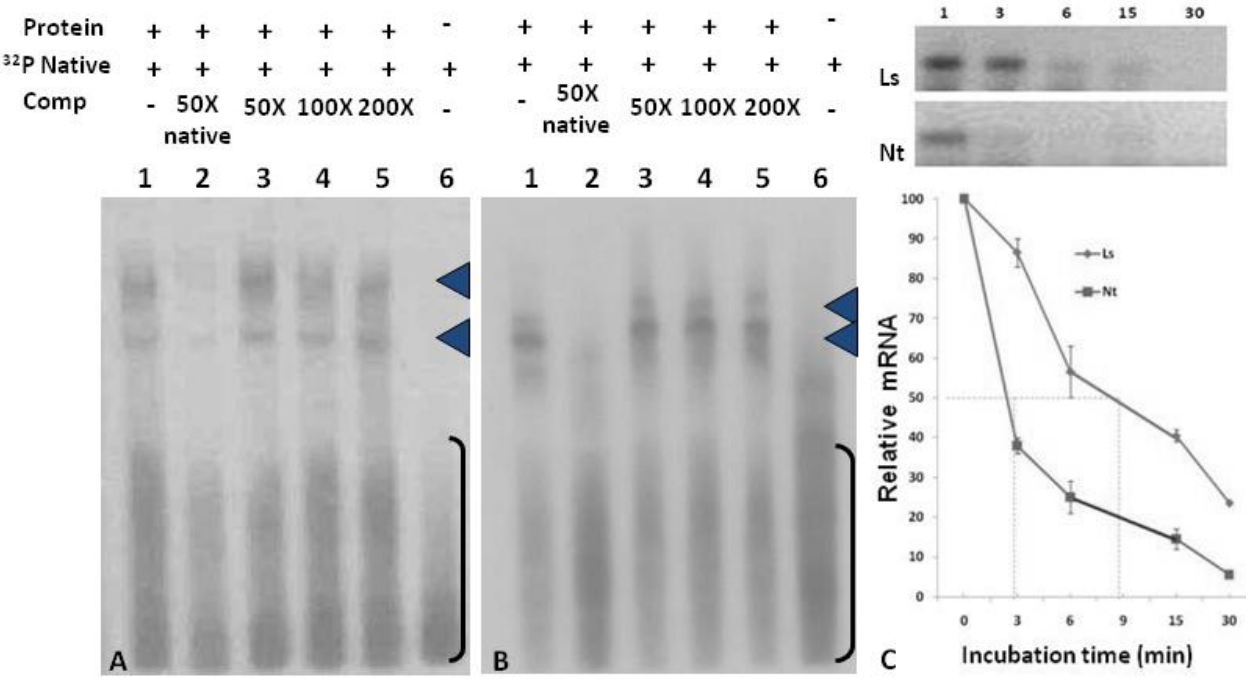


Figure 8: Stromal RBPs preferentially associate with endogenous *psbA* 5' UTR providing transcript stability.
Stromal proteins isolated from *L. sativa* (A) or *N. tabacum* (B) were incubated with radiolabeled native *psbA* 5' UTR (lane 1). Competitions included 50X unlabeled native *psbA* 5' UTR (lane 2) and 50, 100 or 200X unlabeled foreign *psbA* UTR (*N. tabacum* in A, or *L. sativa* in B; lanes 3-5, respectively). Lane 6 shows labeled probe. Brackets indicate free probe, arrows indicate complexes associated with labeled RNA. C) ^{32}P LsUTR-CTB-Pins mRNA was incubated in either Ls or Nt stromal extracts for the time indicated on X axis and extracted RNA was separated by electrophoresis; upper panel shows autoradiographs of the ~800 base UTR-CTB-Pins transcript in each background and (lower) shows graphic representation of two independent experiments. Error bars are standard deviation for two experiments.

Two bands were observed in positive control reactions (lane 1) that were absent in reactions that did not contain stromal proteins (lane 6). The complexes migrated to the apparent molecular weight of 150 and 100 kDa in *L. sativa* extracts and 100 and 80 kDa in *N. tabacum*. The complexes formed between stromal proteins and labeled UTRs were disassociated by 50-fold molar excess of unlabeled endogenous UTR, but not by 50-, 100- or 200-fold excess of unlabeled heterologous *psbA* UTR. This result was consistent over 3 independent experiments in both backgrounds.

To determine if the RBP interaction affected mRNA turnover in these studies, we synthesized labeled Ls*psbA* 5' UTR CTB-Pins transcripts *in vitro*, incubated them in either *L. sativa* or *N. tabacum* stromal extracts and extracted total RNA over time. RNAs were electrophoresed, gels were dried and exposed to film for densitometric analysis. We found the half life of the radiolabeled transcript to be 8.4 min in the homologous *L. sativa* extract while incubation of the transcript in the *N. tabacum* stromal extracts reduced the half life to 2.3 min, a 3.7 fold difference in the rate of turnover (Figure 8C).

Whole genome mapping and analysis

Plastid genome sequences are a valuable tool for the design of plastid transformation vectors and expression constructs as well as phylogenetic analyses and evaluation of whole genome structure. The plastid genome of *D. carota* (Ruhlman et al., 2006) was the first member of the family Apiaceae to be analyzed and published in the public databases facilitating its inclusion in our study of intergenic regions. The

D. carota plastid genome is typical of angiosperms showing highly conserved gene order and quadripartite structure. Plastome monomers comprise 155, 911 bp (Figure. 9). The inverted repeat (IRb and IRa) is 27, 051 bp with the copies separated by two single copy regions. The large single copy (LSC) region is 84, 242 bp in length and the small single copy (SSC) region is 17, 567 bp in length. The predicted coding regions total 136. Of these 115 are unique and 21 are duplicated in the IR. On LSC/IRb boundary, the IR extends into *rps19*, resulting in the duplication of a portion of this gene. There are 81 unique protein-coding genes, 10 of which are duplicated in the IR. Within the IR region is the ribosomal operon, which includes all four rRNA genes as well as tRNA-Ile and tRNA-Ala. There are five additional tRNAs within the IR resulting in a total of 37 tRNA genes, 30 of which are unique. There are 18 genes containing introns, three of which contain two introns (Table 6). Non-coding sequences, including IGS regions and introns, comprise 43.61 % of the *D. carota* plastome. The overall nucleotide composition is 62.34 % AT and 37.66 % GC.

Figure 9. Map of the *D. carota* plastid genome.
The thick lines indicate the extent of the inverted repeats (IRa and IRb), which separate the genome into small and large single copy regions. Genes on the outside of the map are transcribed in the clockwise direction and genes on the inside of the map are transcribed in the counterclockwise direction. Numbered ticks around the map indicate the location of repeated sequences found in the carrot genome; black = direct, blue = palindrome; * indicates that repeated sequence begins at the same position (see Table 7 for details).

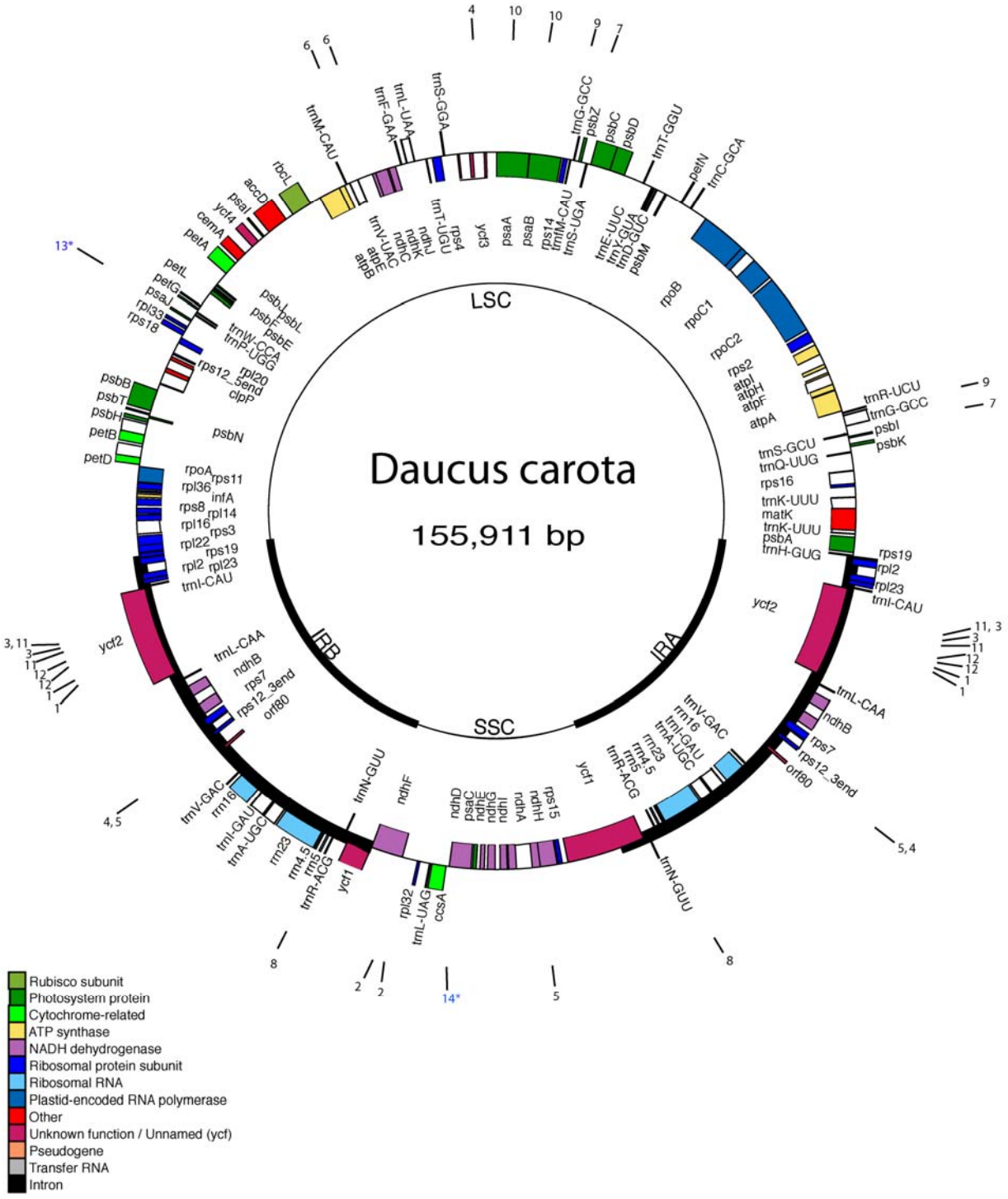

Daucus carota
155,911 bp
LSC
IRB
IRA
SSC
Rubisco subunit
Photosystem protein
Cytochrome-related
ATP synthase
NADH dehydrogenase
Ribosomal protein subunit
Ribosomal RNA
Plastid-encoded RNA polymerase
Other
Unknown function / Unnamed (ycf)
Pseudogene
Transfer RNA
Intron

Table 6. Intron-containing genes found in the *D. carota* plastome.

Protein coding	Transfer RNA
rps16	*trn*-Lys (UUU)
atpF	*trn*-Gly (GCC)
rpoC1	*trn*-Leu (UAA)
yf3+	*trn*-Val (UAC)
clpP+	*trn*-Ala (UGC)*
petB	*trn*-Ile (GAU)*
petD	
rpl16	
*rpl2**	
*ndhB**	
*rps12**+	
ndhA	

* genes are located in the IR; + genes contain two introns

Repeat analysis

Repeat analysis identified 12 direct repeats and two palindromes of ≥ 30 bp with a sequence identity of ≥ 90 % (Hamming distance of 3). Repeated sequences were found in IGS regions, introns and within coding sequence (Table 7). There are four direct repeats in *ycf2*, with repeated sequence ranging up to 70 bp in length.

Table 7. Repeats identified in the *D. carota* plastid genome.
Repeats of at least 30 bp in size, with a sequence identity greater or equal than 90% are included. Repeats marked with * are present in the IR and are therefore duplicated. See figure 9 for location of repeats on the gene map.

Repeat #	Length	Location 1	Location 2
Direct			
1*	70	*ycf2* (IRa,b)	*ycf2* (IRa,b)
2	42	*ndhF*:*rpl32*	*ndhF*:*rpl32*
3*	34	*ycf2* (IRa,b)	*ycf2* (IRa,b)
4*	42	*ycf3* intron 2	*rps12*_3end:*orf80* (IRa,b)
5*	41	*rps12*_3end:*orf80* (IRa,b)	*ndhA* intron
6	34	*ndhC*:*trnV*-UAC	*ndhC*:*trnV*-UAC
7	33	*trnS*-GCU	*trnS*-UGA
8*	31	*rrn5*:*trnR*-ACG (IRa,b)	*trnR*-ACG:*rrn5* (IRa,b)
9	30	*trnG*-GCC	*trnG*-GCC
10	30	*psaB*	*psaA*
11*	30	*ycf2* (IRa,b)	*ycf2* (IRa,b)
12*	30	*ycf2* (IRa,b)	*ycf2* (IRa,b)
Palindrome			
13	30	*psaJ*:*rpl33*	*psaJ*:*rpl33*
14	30	*ccsA*:*ndhD*	*ccsA*:*ndhD*

Upstream sequence variation predominates in plastid genes across taxa.

Sequences of intergenic spacer regions upstream from genes representing different functional groups were extracted from 20 complete plastid genomes representing most major clades of angiosperms, including four grasses and three legumes. Alignments were anchored by the inclusion of 100 bases from the coding

region of adjacent genes. Sequence identity was calculated for the region encompassed by 200 bases upstream of the translation start codon, i.e. promoters and UTRs. For reference and comparison the nucleotide sequence identity was also determined for the aligned coding regions. We found that coding regions across all genera and genes display sequence identity of 80% to 97%, whereas the non-coding regions are 45% to 79%. In keeping with our findings throughout this study employing the *psbA* 5' UTR as an experimental model system we found that despite 95.0% identity in the coding region, identity in the *psbA* upstream region is less than 59% across all taxa (Figure 10). The *cis* feature located at position -49 to -71 in relation to the *N. tabacum* start codon has been shown experimentally to be an important determinant of translation efficiency (Eibl et al., 1999; Zou et al., 2003). Across all taxa sequence identity for this element is 61%; *N. tabacum* and *L. sativa* have 54.2% identity for this region, suggesting that variation in this element may be a contributing factor to reduced foreign protein expression observed in our transplastomic lines. All alignments have been deposited in the Chloroplast Genome Database (http://chloroplast.cbio.psu.edu/).

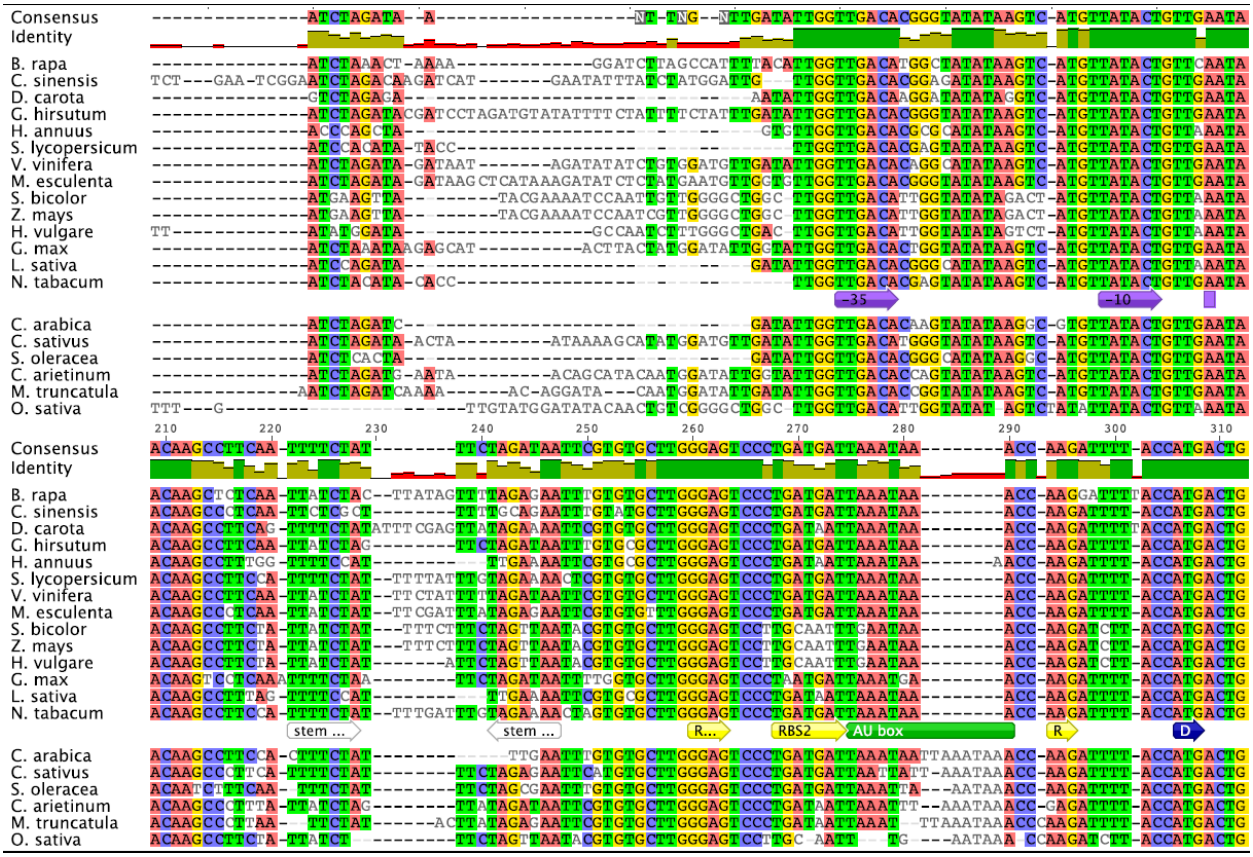


Figure 10: Upstream sequence variation in the *psbA* 5' UTR across taxa.
IGS was extracted from complete plastid genomes of 20 species of angiosperms. Colored bases indicate agreements. Promoter and UTR elements are labeled. Complete data from genomic analyses are available at (http://chloroplast.cbio.psu.edu/)

Discussion

A survey of the literature demonstrates that there is considerable interest in plastid transformation, both in the established system of *N. tabacum* and in crop species that have been more recalcitrant to transformation by this approach. More than 20 reports describe the generation of transplastomic crop plants through the use of species-specific flanking regions to facilitate homologous recombination between the

shuttle vector and the plastid genome. The field has clearly recognized that species-tailoring of recombination sequences can lead to greater transformation potential. Nonetheless, the expression cassettes integrated in these experiments more commonly contain heterologous regulatory elements either from another plant species, usually *N. tabacum*, or a non-plant source. In this study we have utilized wild type *L. sativa* and *N. tabacum* plants along with a suite of transplastomic lines to investigate potential mechanisms that underlie the variation we have observed in foreign protein accumulation resulting from the use of endogenous or heterologous regulatory elements. Our findings suggest that these differences may be influenced at multiple levels including transcription, mRNA processing, mRNA stability, and translation of foreign gene products.

We examined relative abundance of foreign gene transcripts in total RNA extractions of transplastomic lines and found the greatest variation in the monocistron pools in the different CTB-Pins lines. Sequence alignments reveal that there is 100% identity between *L. sativa* and *N. tabacum* in the region of the ribosomal operon P1 promoter required for full PEP activity (-64 to +17; Suzuki et al., 2003). This is supported by similar levels of dicistron accumulation in the *L. sativa* and *N. tabacum* lines where transcription of this mRNA species is driven by endogenous or heterologous P*rrn* (Figure 3). It stands to reason that PEP and its associated transcription factors that rely on recognition sequences within the promoter would be equally responsive to either promoter considering the perfect conservation in these elements. Similarly the core promoter elements shown to be sufficient for developmental regulation of *psbA*

transcription are 90% identical between *L. sativa* and *N. tabacum* (-42 to +9; Hayashi et al., 2003). Transcription of *psbA* in response to light is well established (Klein and Mullet, 1990; Rapp et al., 1992; Chun et al., 2001). Recent findings suggest that light regulated control of transcription from the *psbA* promoter in land plants may operate in part on the red-ox driven phosphorylation of PEP associated trans-acting factors and appears to be independent of additional cis elements (Steiner et al., 2009). Therefore it seems unlikely that the differences we observed in the CTB-Pins monocistron pool result directly from an inability to achieve PEP-mediated synthesis due to divergence in promoter sequence and structure in the heterologous system, i.e. Ls-Nt-CP.

Based on our sequence comparisons the greatest variability upstream of the D1 coding region lies in within the proposed stem-loop structure of the 5' UTR that has been shown to be involved in transcript stability and translation efficiency in land plants (Alexander et al., 1998; Eibl et al., 1999; Shen et al., 2001; Zou et al., 2003). Over our entire sample set this region has a 61% sequence identity, and for *L. sativa* and *N. tabacum* the identity is 54.2%. Our results from polysome analyses indicated that foreign transcripts in *L. sativa* lines which carried the endogenous 5' UTR were more abundant in the upper fractions giving some insight into differences in foreign protein accumulation in the various lines used in this study.

It has been shown previously that the majority of *psbA* transcripts are not polysome associated in *Hordeum vulgare, Spinacia oleracea* and *N. tabacum* (Klein et al., 1988; Minami et al., 1988; Nakamura et al., 1999) and that this population is likely stabilized by RBPs in the stroma (Nakamura et al., 1999, 2001). It was shown by *in*

vitro mRNA degradation assay that turnover of the full length *psbA* transcript was accelerated three fold in *N. tabacum* stromal extracts depleted of RBPs. This result was unchanged when a truncated *psbA* transcript that did not include the 3' UTR was utilized in place of the full length sequence (Nakamura et al., 2001), suggesting that the 5' UTR is involved in the stabilization of *psbA* transcripts in this assay.

A number of studies have investigated the *cis*-elements within the land plant *psbA* 5' UTR to elucidate sequences which are required for the association of RBPs and the role of these interactions in transcript stability, processing and initiation of translation on plastid ribosomes. Using synthetic *psbA* 5' UTRs with specific site mutations and internal deletions it was determined that the stem loop region was a dispensable element as its exclusion did not affect translation of the fused *lacZ* reporter used in one study. The authors identified an AU rich element (AU box) located between RBS1 and RBS2 that together were required and sufficient to initiate translation and proposed a role for the AU box as the primary target sequence for the binding of trans-acting factors (Hirose and Sugiura, 1996). Our sequence analysis, combined with the results from RNA EMSA, suggest that the AU box and adjacent RBS sites are not responsible for differences observed in our experimental system. Between *L. sativa* and *N. tabacum* there is sequence identity of 95% over this region (20 bp), the variation generated by a single base change in RBS2. We did find, however, that this region has diverged in a subset of species, including representatives from each of the clades in our analysis, which have variable TA (AU) insertions at the 3' end of the AU box (Figure 10). Our EMSA analyses employed whole stromal extracts rather than isolated S30 fractions.

Given the many RBPs that have been identified in plastids of land plants and algae it is probable that different factors are involved in the various aspects of mRNA metabolism. While the 30S fractions apparently included RBP factors closely associated with translation initiation, this preparation has potentially excluded a subset of factors responsible for stabilizing transcripts at or near the stem loop structure as well as other determinants that may participate in mRNA turnover.

Subsequent analyses have determined the presence of an endonucleolytic cleavage site that is protected upon the binding of protein factors. This site is localized to the predicted stem-loop region of the *S. oleracea psbA* 5' UTR (-49/-48 relative to start of translation) and the binding interaction is sensitive to the secondary structure of the RNA (Klaff et al., 1997; Alexander et al., 1998). Further studies employing stromal extracts from *A. thaliana* have confirmed temperature dependence of the RBP binding interaction and demonstrated that the interaction is ablated under oxidizing conditions (Shen et al., 2001). Additional support for the role of the stem loop structure in stabilization of transcripts that include the *psbA* 5' UTR is provided from transplastomic studies. *Nicotiana tabacum* plants were generated that express *uidA* (GUS) mRNA from chimeric genes where transcription was driven by P*rrn* with the full length endogenous *psbA* 5' UTR (85 bases) or deletion variants upstream of the GUS coding region. In all experimental constructs mRNA abundance was reduced compared to the control despite transcription from identical promoters (Zou et al., 2003).

Although there may be other sequence elements that could be responsible for the marked differences in foreign protein accumulation we have observed in these

studies, our evaluation of sequence identity within the promoters and *psbA* 5' UTRs used in the lines suggest the stem loop region of *psbA* 5' UTR and its effect on transcript stability as the most likely candidate. We included the full length, highly conserved ribosomal operon promoter which has been shown to be regulated both by development and light (Baumgartner and Mullet, 1991; Baumgartner et al., 1993; Vera and Sugiura, 1995; Courtois et al., 2007). It is plausible that P*rrn*-driven transcription proceeds in this manner within those lines that carry the heterologous promoter but the effect is only mildly detectable in terms of protein accumulation due loss of RBP interaction for transcript stability. Given that similar light and developmental variation is seen in Ls-g10-CP lines, which should be independent of such regulation, this is probably a more general phenomenon related to the overall functioning of the plastid at a given sample point. We were able to quantify respectable yields of foreign protein, up to 3% and 17% CTB-Pins in TLP from mature leaves of Ls-Nt-CP and Ls-g10-CP lines respectively, testifying that conserved Shine-Delgarno type ribosome binding sites are sufficient for translation in plastids. Also, the abundance of di- and polycistrons identified in lower sucrose gradient fractions confirmed that these transcript species were amenable to translation as previously demonstrated (Quesada-Vargas et al., 2005). We considered the possibility that there may be some difference in turnover at the protein level that limited accumulation in the lower expressing lines. The relatively massive accumulation of CTB-Pins in older tissues of all the lines investigated demonstrates that this protein is highly stable in plastids. Furthermore these plants all express the identical CTB-Pins in terms of amino acid sequence.

RNA EMSA assays using wild type stromal extracts demonstrated that the heterologous *psbA* 5' UTR was not an effective competitor for binding factors that may be involved in transcript stability, and we observed that the half life of the foreign transcript was reduced by up to 3.7 fold in the heterologous background. We attribute this strongly preferential binding of the endogenous UTR to the stem loop structural element as this is the only region where we have found significant sequence variation between *L. sativa* and *N. tabacum*. Conceivably, *in planta,* the ability of foreign gene transcripts equipped with heterologous UTR elements to compete for stabilization factors would be hampered by the presence of abundant wild type *psbA* transcripts leading to rapid turnover of foreign RNA species in the plastid. The persistence of the dicistron pool in heterologous lines may indicate that these chimeric transcripts are inherently more stable, perhaps due to their size or some structural characteristic yet to be identified.

Previous reports from our lab and others have used densitometric quantitation of foreign transcripts visualized by northern blotting of total RNA with autoradiography. Based on these results it has been proposed that transcript abundance is not related to foreign protein accumulation in the lines under investigation, yet this methodology does not permit consideration of the more subtle factors at work in mRNA metabolism in plastids. In these studies comparisons have been drawn to evaluate the contribution of transcription in light induced synthesis of foreign protein where its expression is driven by *psbA* 5' UTR (Staub and Maliga, 1994; Fernandez-San Millan et al., 2003; Dhingra et al., 2004). While it is clear that transcript ratio from light to dark could not account for

the rapid increase in corresponding foreign protein, perhaps it was the more qualitative aspects of the transcript pool that participated in this response, and there may exist a cycle of transcription and turnover that is overlooked when measures are taken of total mRNA abundance.

Here we have evaluated the species-specific nature of protein factor binding to the *psbA* 5' UTR as a mechanism influencing foreign protein accumulation in transplastomic lines. We found that exchange of the full length UTR between *L. sativa* and *N. tabacum* resulted in a reduction in CTB-Pins expression of at least 97%. This was the case in young and mature leaves sampled at four time points. CTB-Pins accumulated much higher levels in older, but not senescent, leaves of these *L. sativa* plants but this could not compensate for the differences in overall expression as accumulation still showed a reduction of approximately 85% compared to *N. tabacum* plants with the endogenous UTR construct.

We estimate that CTB-Pins in fully expanded leaf tissue to comprised 57% to 58% of the total leaf protein when harvested near the end of the light period and reached as high as 72% in leaves collected further down on the stem. Unlike a recent report of massive accumulation of an antimicrobial in transplastomic *N. tabacum* (Oey et al., 2009) we saw no deleterious phenotype, perhaps owing to the observation that this level of foreign protein accumulated over time in our plants. The use of *N. tabacum* P*rrn* and g10 in transformation constructs was highly successful in the preceding example which should remind us that each foreign gene construct needs to be assessed independently. This regulatory combination has not yielded such remarkable

levels of expression for other proteins in *N. tabacum* or any other species in which it has been utilized. A recent example is the very low expression level in *N. tabacum* for a diphtheria-pertussis-tetanus vaccine using P*rrn* and g10 (Soria-Guerra et al., 2009). The use of endogenous *psbA* 5' UTR has led to the accumulation of numerous gene products in transplastomic *N. tabacum* (Verma et al., 2008), including proteins that had been previously unattainable at satisfactory levels using this technology (Fernandez-San Millan et al., 2003; Dhingra et al., 2004). We have now generated transplastomic *L. sativa* plants that accumulate abundant foreign protein. While the expression of first therapeutic protein produced in *L. sativa* was accomplished using the *N. tabacum* P*rrn*/g10 system for expression of the gene of interest (Ruhlman et al., 2007), the implementation of *L. sativa* specific regulatory elements has contributed to the development of a highly reproducible transformation system that generates transplastomic *L. sativa* plants expressing foreign proteins to high levels (Ruhlman et al., manuscript in preparation).

There are a great many factors to consider when designing transformation constructs for the generation transplastomic plants, particularly when the target is a new species for which there is no standardized approach. The use of species-specific integration sequences, codon optimization for plastid expression and the inclusion of N-terminal stabilization sequences have been essential to the development of novel transplastomic lines. The emergent study of pentatricopeptide repeat (PPR) proteins in addition to well established research on RBPs have revealed that the interactions between protein factors and their cognate RNA sequences is highly specific. Our

evaluation of UTR sequences from taxonomically diverse species for genes representing the various functional groups found in plastids, combined with our experimental findings using the *psbA* 5 UTR in particular, argues for the use of species-specific regulatory elements for the accumulation of foreign protein in transplastomic plants.

AUTOIMMUNE THERAPY UTILIZING ORALLY DELIVERED PLASTID-DERIVED SELF ANTIGEN

Introduction

Type one diabetes mellitus (T1D) is an autoimmune disease resulting from the T-cell mediated destruction of the insulin producing ß-cells of the pancreatic islets. T1D is estimated to affect 246 million people worldwide with up to a million cases in the United States alone. Most commonly diagnosed before puberty, the incidence of T1D is higher than that of all other chronic childhood diseases and is thought to be increasing. Complications associated with T1D include eye and skin dysfunctions, heart disease and stroke, nephropathy and diabetic neuropathy and contribute to the nearly $100 billion financial burden faced in the US. Extensive research effort has focused on induction of immune tolerance *via* presentation of islet autoantigens at mucosal surfaces, particularly in the gut.

Within the small intestine, more densely concentrated in the lamina propria of the ileum, are lymphoid foci called Peyer's patches that facilitate the development of immune responses in the gut mucosa. Large numbers of intraepithelial lymphocytes participate in the response including T-cells, B-cells and antigen presenting cells (Sercarz et al., 1989; Faria and Weiner, 2005). Given the variety and abundance of dietary proteins and commensal microorganisms found in the vicinity of these immune cell populations it is expected that mechanisms exist to subdue responses to antigenic stimulation. This phenomenon, referred to as peripheral tolerance (Sercarz et al., 1989), is mediated in part by regulatory T cells (Treg). Treg comprise a population of

the immune system that is distinct from T effector (Teff) cells and consists of several subclasses. Two broad classifications are the natural Treg, differentiated in the thymus, and the adaptive Treg which acquire their regulatory phenotype in the periphery. Treg express CD4 and CD25 (α-chain IL2 receptor) on their surface and it is the expression of CD25 and the transcriptional regulator Forkhead-box Protein 3 (FoxP3; Fontenot et al., 2003; Hori et al., 2003) that primarily distinguishes them from the effector population which is CD25 and FoxP3 negative. CD4+CD25+ Treg have demonstrated roles in immune tolerance to autoantigens including T1D *via* active suppression of Teff with islet antigen specificities (Homann and von Herrath, 2004; Suri-Payer and Fritzsching, 2006). This suppression appears to be mediated in part by dendritic cells (DC) which are abundant in the gut associated lymphoid tissues (GALT). Reports indicate that retinoic acid (RA), the active metabolite of vitamin A produced by CD103+ DC in the gut, induces Treg differentiation from naïve CD4+CD25- T cells (Kang et al., 2007; Sun et al., 2007) and that lymphocytes primed by gut DCs in the presence of RA are imprinted for gut homing (Iwata et al., 2004; Mora et al., 2006).

Studies aimed at induction of tolerance in autoimmune models have established the potential of Treg for amelioration of pathogenic progression (Sun et al., 2006; Sun et al., 2007). For T1D, oral delivery of islet autoantigens such as proinsulin/insulin and glutamic acid dehydrogenase (GAD), has resulted in positive outcomes for NOD mice in terms of delayed onset diabetes, as yet this success has not been reported in human trials (Chaillous et al., 2000; Pozzilli et al., 2000; Skyler et al., 2005).

Antigen expression in nuclear transgenic plants has been investigated as a means to deliver effective doses to the gut mucosa *via* ingestion of plant tissues (Walmsley and Arntzen, 2003; Arntzen et al., 2005). A limitation in this approach for autoantigen delivery lies in the low level of foreign protein accumulation that has been achieved in stable transformants (Doran, 2000; Daniell et al., 2005). Although several human clinical trials utilizing transgenic plant tissues for oral vaccination (Tacket, 2007) have yielded positive results, studies aimed at induction of oral tolerance have not seen the same success. While expression levels suitable for oral tolerance induction in mice have been reported, the challenge of clinical relevancy has not been met until recently.

Plastid transformation has numerous advantages (Singh et al., 2009), among them the demonstrated capacity to produce transplastomic plants that accumulate antigens and other therapeutic proteins within the range of application in human trials utilizing minimally processed leaf material (Daniell et al., 2001; Tregoning et al., 2003; Watson et al., 2004; Molina et al., 2005). The efficacy of employing transplastomic plants expressing CTB fusions to facilitate receptor mediated transmission across the gut epithelia (Limaye et al., 2006) allows the potential to deliver adequate doses to submucosal layers for exposure to lymphoid structures. Several investigations have demonstrated efficacy in animal models using transplastomic *N. tabacum*-derived purified proteins, as well as oral delivery approaches (Koya et al., 2005; Arlen et al., 2007; Chebolu and Daniell, 2007; Ruhlman et al., 2007; Arlen et al., 2008; Davoodi-Semiromi et al., 2009, Rosales-Mendoza et al., 2009; Soria-Guerra et al., 2009). Ultimately, to translate the promise of transplastomic technology to successful human

clinical trials on oral delivery of autoantigens, a non-toxic edible platform must be employed.

Transplastomic *L. sativa* (Lelivelt et al., 2005; Kanamoto et al., 2006; Ruhlman et al., 2007) has become an efficient platform for oral delivery of antigens in edible leaves. Since our previous report on the expression of CTB-Pins in transplastomic *L. sativa*, the material used in the present study, we have established numerous highly-expressing biopharmaceutical lines (Davoodi-Semiromi et al., 2009). Further analyses of antigen accumulation in CTB-Pins line L100 indicate that these plants express at levels much higher than previously determined. We have used oral delivery of L100 lettuce to evaluate its efficacy for induction of tolerance by looking at the effect on Treg suppressive capacity and ultimately at onset of diabetes in NOD mice.

Materials and Methods

Confirmation of non-Mendelian inheritance, production and harvest of *L. sativa* leaves for animal studies

Seed collected from primary transplastomic *L. sativa* g10 line (L100; Ls-g10-CP described in previous chapter) was germinated on MSO medium solidified with 5.8 g L^{-1} Phytablend® (Caisson) with 200 µg mL^{-1} spectinomycin dihydrochloride (Sigma) along with wild type seeds to confirm transmittance of transgenes to progeny. Plants were hardened in peat pots and transferred to the green house along with wild type *L. sativa* plants. Mature leaves were harvested from 80 transplastomic individuals yielding ~5.2 kg of fresh weight. Intact leaves were washed, midribs were removed and materials

were stored in half-kg batches at -80 ^{o}C for approximately 10 months. Prior to initiation of *in vivo* study 3.5 kg of transplastomic *L. sativa* (1.5 kg of wild type) was removed from storage and processed under liquid nitrogen to produce a homogenous single batch which was then further ground to a fine powder, aliquoted for weekly doses and returned to -80 ^{o}C. Aliquots were removed from storage and homogenized with the addition of 41 µL 20X PBS for each gram of ground tissue.

GM_1 binding assay

In order to test the ability of *L. sativa* plastid derived CTB-Pins to bind to GM_1 receptor a CTB-GM_1 binding assay was performed. *Lactuca sativa* extracts were prepared in protein extraction buffer as for western blotting excluding DTT (previous chapter). Ninety-six well plates were incubated with monosialoganglioside-GM_1 (Sigma) (3.0 µg/ml in bicarbonate buffer) and bovine serum albumin (BSA; control) overnight at 4°C. Plates were washed three times each with PBS plus 0.1% Tween 20 (PBS-T) and sterile water, the plate was blocked with 0.25% BSA in PBS for one hour at 37 °C. CTB standards (Sigma) and soluble protein extracts were diluted in extraction buffer (without Tween 20 and PMSF). The standards and samples were then added in duplicate and incubated at 4°C overnight. Rabbit α-cholera toxin primary antibody (1:8000 *N. tabacum*, 1:3000 *L. sativa*; Sigma) and horseradish peroxidase conjugated donkey anti-rabbit secondary antibody (1:12 500; Biomeda Corp., Foster City, CA) was used to detect the binding of CTB-Pins to GM_1 receptor. The plate was washed three times with both PBS-T and sterile water, and 100 µl of TMB (3,3,5,5-tetramethylbenzidine) soluble

solution substrate (American Qualex, San Clemente, CA) was added to the wells and incubated in the dark for 30 min. The reaction was stopped with 50µl 2M H_2SO_4, and then read on a plate reader (Dynex Technologies, Chantilly, VA) at 450 nm.

Preparation of doses

Dose preparation for 15 (+15%) mice was established at the onset of the study and maintained until the treatment was terminated. *Lactuca sativa* leaf tissue, wild type or expressing CTB-Pins, was homogenized in 20X PBS and distributed into doses as shown in figure 11.

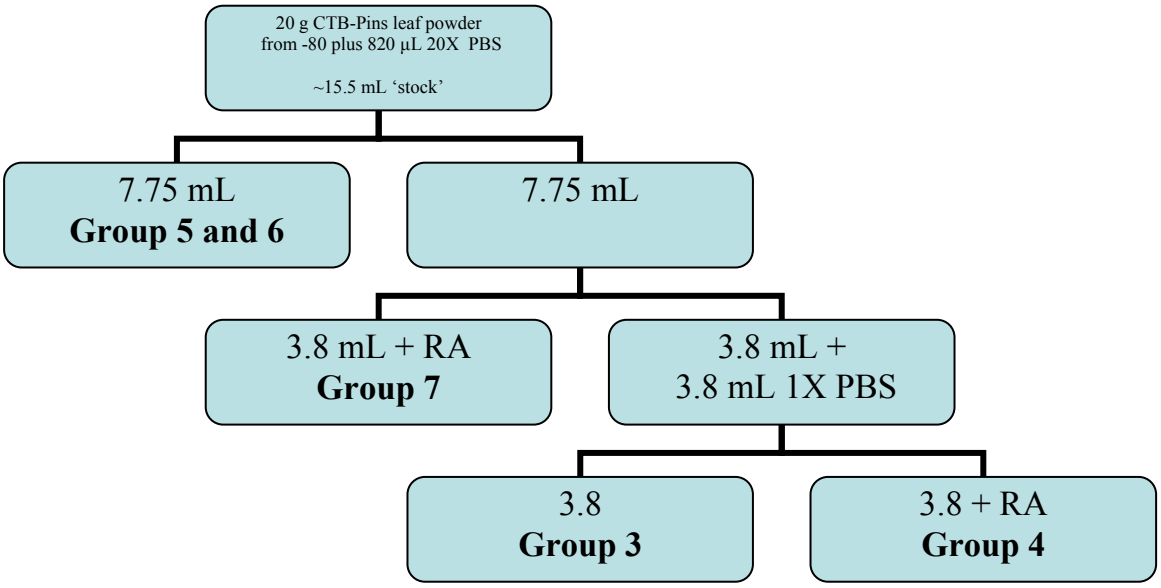


Figure 11: Flow chart showing preparation of doses for oral delivery.
Doses were prepared in the laboratory and transported on ice to the animal facility. RA stock was prepared in 1X PBS, 600 µL was added where indicated to supplement the whole group.

Preparations were packed in ice and transported immediately to the animal facility for oral gavage. Leaf homogenates were packed in one mL syringes. Each animal

received 200 µL of homogenate delivered by oral gavage into the stomach through a 1.5 inch, ball-tipped 20 gage needle (Popper & Sons, INC, New Hyde Park , NY).

Dose administration

For tolerance induction studies 105 female non obese diabetic (NOD/Shi-LtJ) mice were from acquired at four weeks of age (here after referred to as week 4 of study) from Jackson Laboratories (Bar Harbor, ME). Mice were housed at the University of Central Florida wild animal facility in ventilated cages under specific pathogen-free conditions.

Table 8: Dose regime:
Estimated dose of leaf material (mg) and CTB-Pins (µg) delivered to each mouse.

Group	mg leaf material	CTB-Pins µg supernatant	CTB-Pins µg homogenate	Frequency ($week^{-1}$)
1) untouched	na	na	na	na
2) wild type L.s.	233	na	na	1
3) low dose	117	23	211	1
4) low dose RA*	117	23	211	1
5) Intermediate	233	47	419	1
6) high dose	233	47	419	2
7) high dose RA*	233	47	417	2

* Groups 4 and 7 received 102 µg retinyl acetate supplement once per week.

All animal protocols were conducted in accordance with UCF Institutional Animal Care and Use Committee approval. Upon receipt mice were randomly assigned to cages. Each cage contained 5 animals and 3 cages were designated to each treatment group (n=15). Autoclaved Harlan Teklad LM-485 chow (Harlan, Madison, WI) and autoclaved water were available to all animals *ad libitum* for the duration of the study.

Doses of *L. sativa* leaf homogenates were administered beginning in week 5 as outlined in table 8.

Glucose monitoring

All groups were screened twice per week starting at week 12 by testing for the presence of glucose in the urine using Clinistix® strips (Bayer HealthCare, Tarrytown, NY, USA). In the case of positive urine test, blood was drawn by tail vein puncture and blood glucose was measured using an ACCU-CHEK® Aviva blood glucose meter and test strips (Roche Diagnostics Corporation, Indianapolis, IN) Mice were considered diabetic following two consecutive tests resulting in blood glucose level greater than 250 mg dL^{-1}. To ascertain that orally delivered CTB-Pins did not have an effect on glucose metabolism 10 pre-diabetic animals were tested immediately before and one hour after gavage in week 17.

Preparation of splenocytes

Mice were euthanized (Table 9) by inhalation of isofluorane followed by cervical dislocation and dissected along the midline through the peritoneum to open the abdominal cavity. The incision was extended laterally to the fore and hind limbs and the skin reflected to permit access to the viscera. Spleens were removed, sections were placed in ~ 500 µL sterile RPMI with 25 mM HEPES and 2.1 mM L-glutamine (HyClone, Logan, UT) supplemented with 10% fetal calf serum and 100 µg mL^{-1} penicillin and streptomycin (called RPMI hereafter) and held on ice. Spleens of individual animals were forced through 70 µm strainers (BD Biosciences, San Jose, CA) along with the

media. The volume of single cell suspensions was adjusted to 5 mL with RPMI. For proliferation assay cells were centrifuged for 10 min, 4 ^{o}C, 1, 160 x *g* in a swinging bucket rotor (Beckman GS-6R, Beckman Coulter). Cell pellets were resuspended in one to 2 mL of erythrocyte lysis buffer (10 mM $KHCO_3$. 150 mM NH_4Cl, 0.1 mM EDTA, pH 7) and incubated at 22 ^{o}C for 5 min. Centrifugation was repeated following the addition of five volumes of RPMI. Resulting cream to pink colored pellet was thoroughly resuspended in 2 mL RPMI. All lymphocyte suspensions were diluted tenfold in 0.04% trypan blue (Sigma, T8154) and unstained cells were enumerated with a haemocytometer (Hausser Scientific, Horsham, PA).

Table 9: Sacrificed animals by week.

Week	10	18	20	21	23	24	26
1) untouched	3	1	2	0	0	2	0
2) wild type L.s.	3	0	3	2	0	2	2
3) low dose	3	1	3	0	0	0	0
4) low dose VA*	2	1	1	2	0	0	0
5) Intermediate	2	1	0	4	0	1	0
6) high dose	0	0	0	0	4	2	1
7) high dose VA*	2	0	0	0	1	0	3
total	15	4	9	8	5	7	6
54 animals							

Proliferation assay

Erythrocyte-cleared splenocyte suspensions (whole lymphocytes) were adjusted to 2 X 10^6 cells mL^{-1} with RPMI. To assay for the proliferative potential of splenocytes from individual animals, 2 x 10^5 cells were seeded into wells of a round bottom Costar® microtiter plate (Corning Inc., Corning, NY). For each sample four culture conditions were established in triplicate wells in a total volume of 200 µL. Lymphocytes were

unstimulated or activated by addition of 2.5 μg mL^{-1} each BD Pharmingen™ anti-CD3ε and anti-CD28 (#553057 and #553294; BD Biosciences). For non-specific activation 2.5 μg mL^{-1} T-cell mitogen concanavalin A (conA; Sigma) was used. For antigen-specific activation 20 μg mL^{-1} recombinant human insulin suspension was added (I9278; Sigma). Cells were cultured for 48 hours at 5.5% CO_2, 37 ^{o}C in a humidified, water jacketed incubator (Forma Scientific, Marietta, OH). Proliferation was evaluated based on the incorporation of 6-^{3}H-thymadine. Fifty microliters of culture supernatant was removed from each well and replaced with RPMI containing 1 μCi 6-^{3}H-thymadine (PerkinElmer). After 16 hours incubation, cells were harvested by vacuum deposition onto filter paper mats. Mats were cut into blocks representing individual wells and suspended in Scintiverse™ BD Cocktail (Fisher Scientific, Pittsburgh, PA). Activity in each sample was measured by scintillation counting using a Packard Tri-Carb 3100TR liquid scintillation analyzer with QuantaSmart™ Version 1.31 software (PerkinElmer).

Suppression assay

To evaluate the suppressive capacity of CD4+CD25+ regulatory T-cells isolated from diabetic NOD mice, single cell suspensions of whole splenocytes were magnetically labeled using the mouse CD4+CD25+ Regulatory T-cell isolation kit, the OctoMacs™ separation unit and MS columns (Miltenyi Biotec Inc, Auburn, CA) according to manufacturer's instructions. Isolated CD4+CD25- (Teff) and CD4+CD25+ (Treg) cells were diluted tenfold in trypan blue, unstained cells were enumerated as described above and suspensions were adjusted with RPMI to contain 1.0 x 10^{5} cells

mL^{-1}. For co-culture, the Teff population was fixed at 5 X 10^4 cells per well while Treg were present in equal (1:1) or decreasing proportion (1:0.5 or 1:0.25). Control wells included 5 X 10^4 Teff or Treg. Proliferation of the responding population was assessed on the basis of 6-^{3}H-thymadine incorporation as described for proliferation assay.

Results

GM_1 binding assay

Soluble extracts of T_0 transplastomic *L. sativa* lines were analyzed by the GM_1 binding assay to evaluate formation of functional pentamers from the CTB-Pins monomers. Results demonstrated that pentameric structures of CTB-Pins were formed. CTB can bind the GM_1 receptor only when in the pentameric conformation (Merritt *et al.,* 1994), therefore this assay confirmed the correct three dimensional configuration and disulfide bond formation of CTB within transgenic plastids (Figure 12).

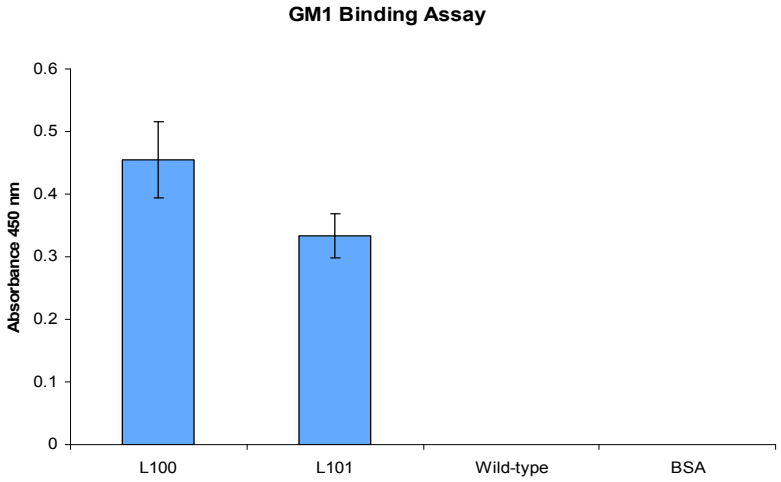
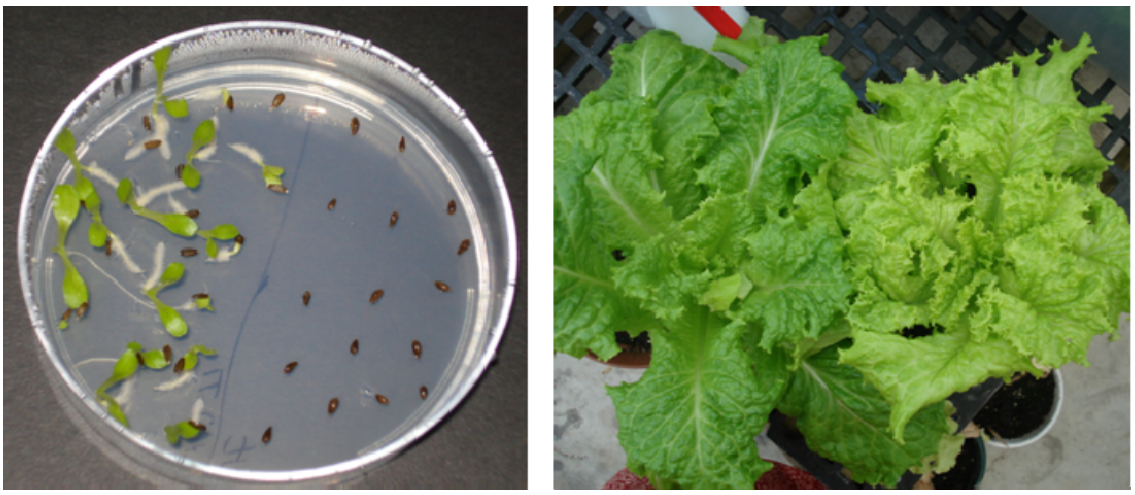


Figure 12: GM_1 binding activity in *L. sativa* transplastomic lines.
Soluble extracts from CTB-Pins *L. sativa* lines were incubated in plates coated with the GM_1 receptor. Transplastomic lines and controls are indicated on the x-axis.

Production and harvest of *L. sativa* leaves for animal studies

Seeds of transplastomic *L. sativa* line L100 germinated and greened uniformly on spectinomycin confirming non-Mendelian inheritance of transgenes (Figure 13A). Plants were grown in the UCF greenhouse along with wild type plants. Mature leaves were harvested from 80 transplastomic individuals yielding ~5.2 kg of fresh weight. Transplastomic plants were similar in appearance and yield to wild type plants (Figure 13B).



A **B**

Figure 13: Confirmation of non-Mendelian inheritance and production of T1 CTB-Pins *L. sativa*.
A) Seed from T0 plants grown on spectinomycin media. B) Wild type (left) and T1 transplastomic (right) plants.

CTB-Pins expression in Transplastomic *L. sativa*

Construction of transformation vectors, generation and confirmation of transplastomic *L. sativa* line L100 is detailed in Ruhlman et al. (2007). We previously reported accumulation of CTB-Pins in line L100 as approximately 2% of TSP or ~13.6

µg CTB-Pins per 100 mg of ground mature leaf tissue. During the course of our evaluation of the dosing format we found this estimate represents a fraction, (less than 10%) of the foreign protein present in our sample (Figure 14). Densitometric analysis of leaf protein homogenates revealed that CTB-Pins accumulation had reached more than 12.5% of the total protein in mature leaves (up to 200 µg/100 mg ground tissue). Similarly we found that *N. tabacum* lines contained more than 5 times the level of CTB-Pins than we had previously estimated when protein was evaluated in homogenates.

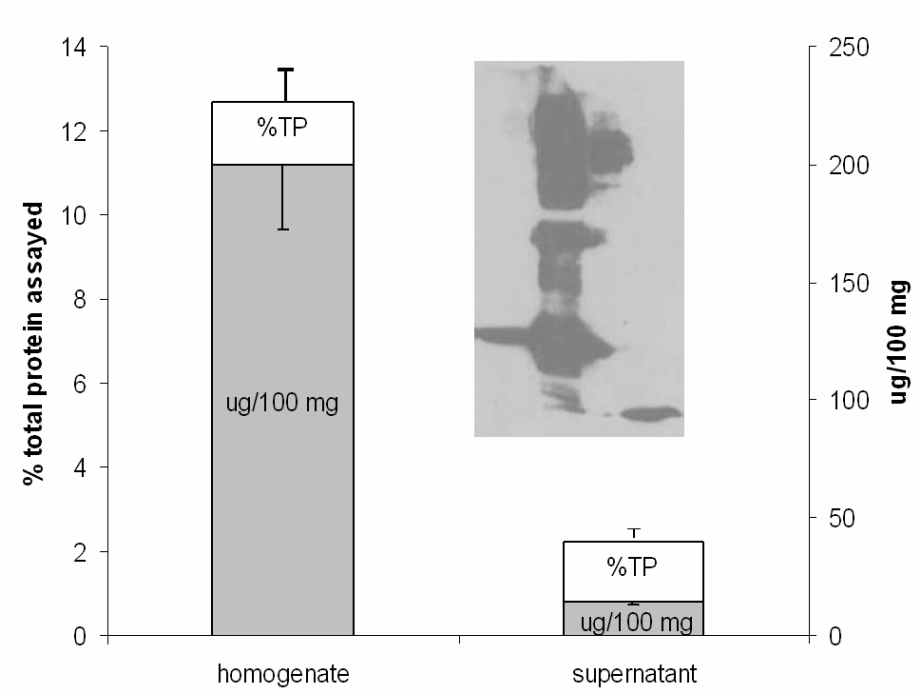


Figure 14: The majority of CTB-Pins accumulation in mature leaves of *L. sativa* was present in the insoluble fraction.
CTB-Pins accumulation is represented as % of total protein assayed (left axis; empty bars) and µg of CTB-Pins per 100 mg ground leaf tissue (right axis; shaded bars). Inset: western blot probed with α-CTB, left to right: 30 µg total soluble protein (supernatant); 30 µg total protein (homogenate); markers; 100 ng CTB standard.

Glucose monitoring

Beginning in week 12, animals were monitored twice per week for the presence of glucose in the urine. Upon detection of urine glucose, a blood sample was analyzed for glucose content. Animals were considered diabetic when two consecutive readings over two weeks were above 250 mg dL^{-1}. Week 30 was taken as the endpoint of the study. If a mouse expired incidentally without being diagnosed as diabetic its number was taken out of the calculation for percent diabetic. Delay in the onset of diabetes was observed in group 7 (high dose w/ RA) relative to both low dose groups, wild type treated and untouched control. While group 6 (high dose) individuals experienced onset earlier in the study period at the endpoint both group 6 and 7 reached the same overall percent diabetes. In group 4 (low dose w/ RA) onset of diabetes was not significantly delayed but by week 30 this group had the greatest proportion of diabetes-free individuals. At week 30 groups 2, 3 and 5 were all 8% diabetes free (Figure 15). To ascertain that orally delivered CTB-Pins had no effect on glucose metabolism in the mice we analyzed ten group 7 individuals before and one hour after gavage in week 17.

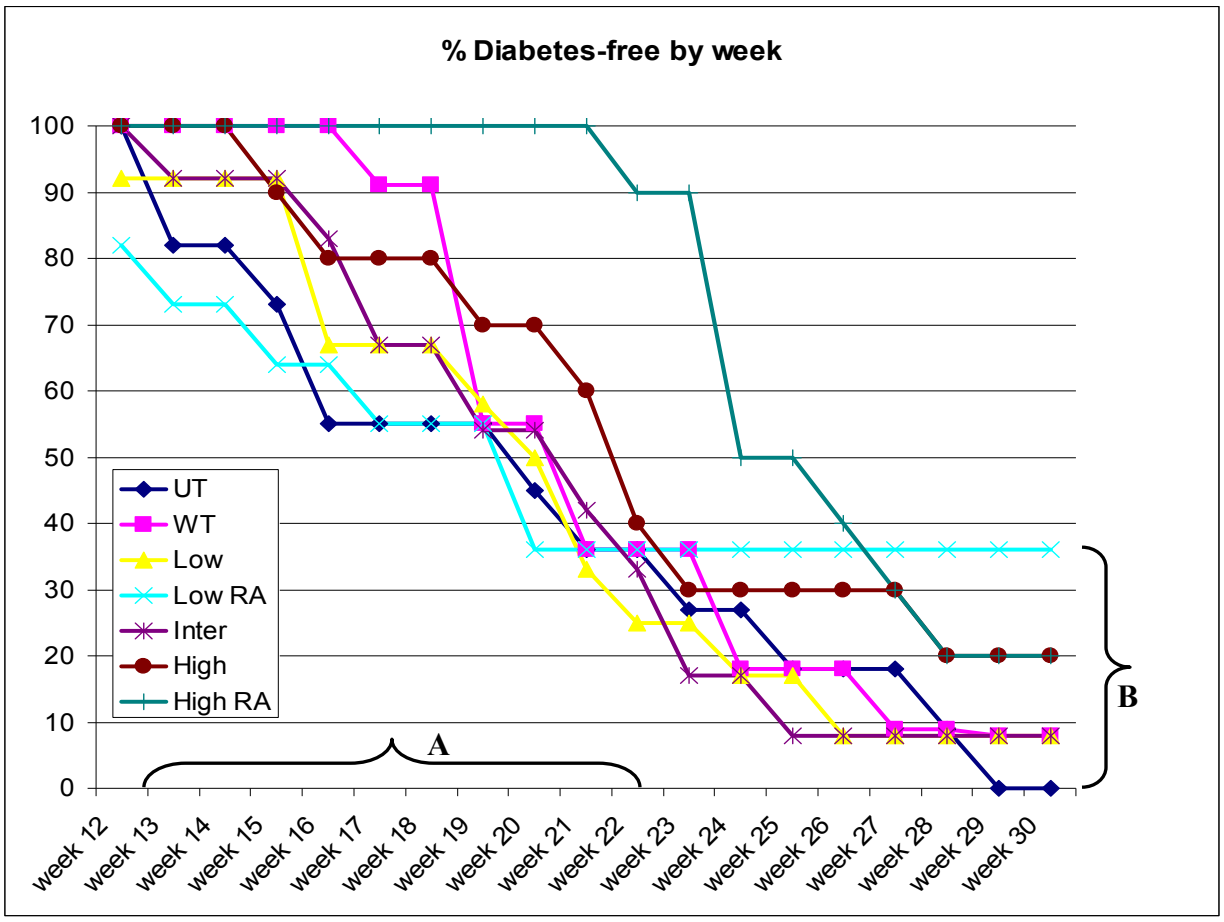


Figure 15: Effect of orally delivered CTB-Pins on percent diabetes by week. Number of mice free of diabetes in week 30 (endpoint) was: group 1) 0/11; group 2) 1/11; group 3) 1/12; group 4) 4/11; group 5) 1/12; group 6) 2/10; group 7) 2/10. Bracket indicates A) significant delay of onset; B) significant difference at endpoint ($P<0.05$) determined by Mantel-Cox Log-Rank test.

At that time no group 7 individuals were diabetic. As determined by blood glucose level we found that oral delivery of transplastomic *L. sativa* did not have a detectable metabolic effect (Table 10).

Table 10: Effect of oral gavage on glucose metabolism.
Blood glucose values for 10 individuals in group 7 in mg dL^{-1}

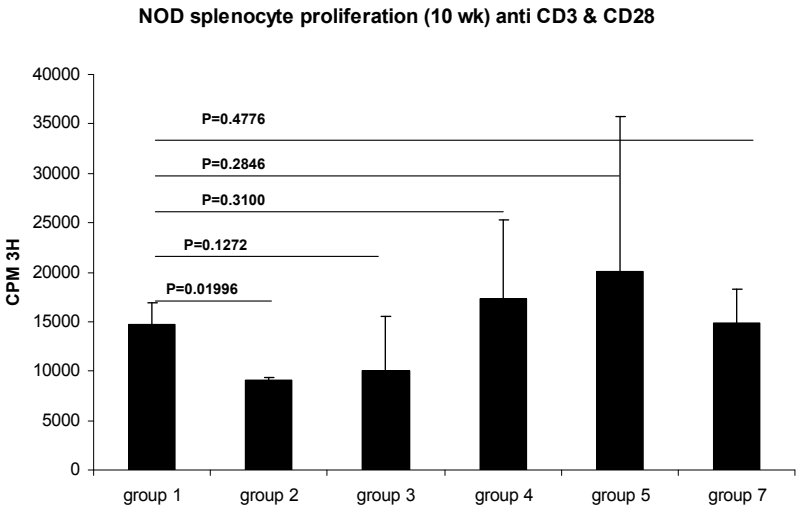
Before	103	94	92	74	126	147	137	103	110	88
After	96	119	85	87	128	137	140	141	107	111

Splenocyte assays

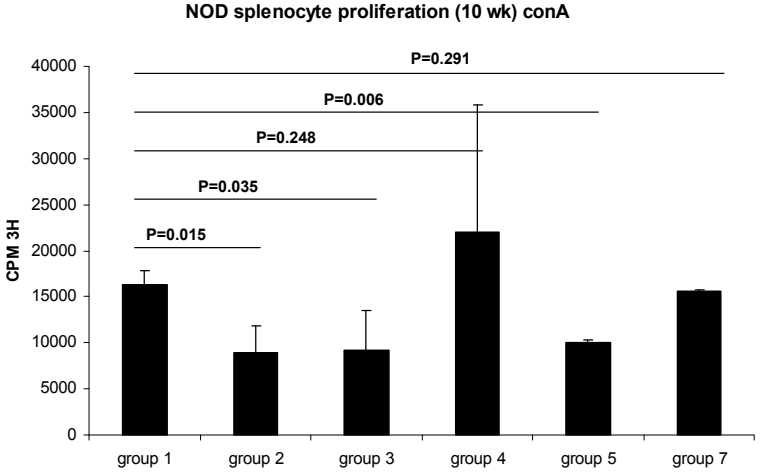
Spleens of NOD mice from different treatment groups were removed and single cell suspensions were prepared as described in materials and methods. Erythrocyte depleted splenocytes were analyzed for proliferation in response to T-cell receptor ligation via α-CD3ε and α-CD28 antibodies, incubation with T-cell mitogen conA and with human insulin. Splenocyte suspensions were magnetically separated to isolate distinct populations of CD4+CD25- (Teff) and CD4+CD25+ (Treg) cells. Defined numbers of each population were co-cultured with α-CD3ε and α-CD28 antibodies and the suppressive capacity of Tregs was evaluated.

At week 10 of the study, mice were euthanized to determine the extent of T-cell infiltration of the pancreas. At this time all mice were urine glucose negative and therefore not considered to be diabetic. Mice from group 6 (high dose) were not included as we had five incidental losses in this group by week 10. Figure 16 shows results from splenocyte proliferation assay. Each sample well was seeded with 2 $x10^5$ splenocytes and cultured for 72 hours in the presence of the indicated additive. During the last 16 hours of culture cells were exposed to ^{3}H-thymidine and incorporation into the DNA was measured by scintillation counting. There was no difference in proliferation between groups in response to activation with α-CD3ε and α-CD28 (Figure 16A).

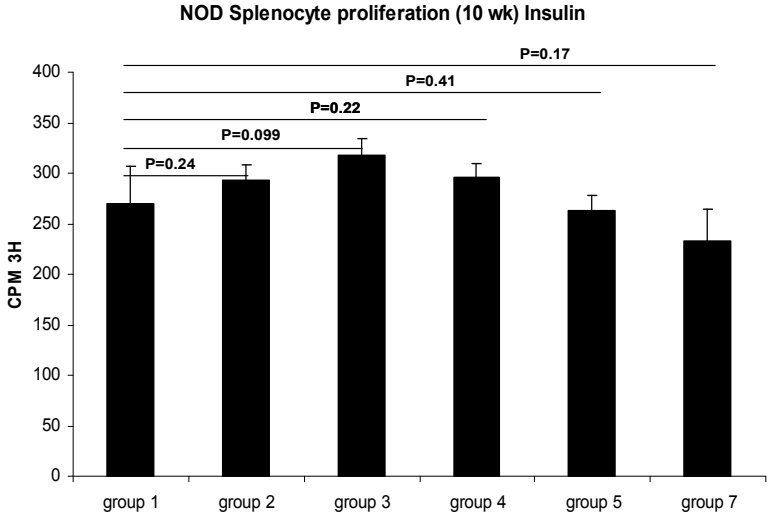
Figure 16: Week 10 splenocyte proliferation was similar across all groups.
2 X 10^5 NOD splenocytes were incubated with A) α-CD3ε and α-CD28; B) conA or C) human insulin


NOD splenocyte proliferation (10 wk) anti CD3 & CD28
CPM 3H
40000
35000
30000
25000
20000
15000
10000
5000
0
P=0.4776
P=0.2846
P=0.3100
P=0.1272
P=0.01996
group 1
group 2
group 3
group 4
group 5
group 7

A


NOD splenocyte proliferation (10 wk) conA
CPM 3H
40000
35000
30000
25000
20000
15000
10000
5000
0
P=0.291
P=0.006
P=0.248
P=0.035
P=0.015
group 1
group 2
group 3
group 4
group 5
group 7

B


NOD Splenocyte proliferation (10 wk) Insulin
CPM 3H
400
350
300
250
200
150
100
50
0
P=0.17
P=0.41
P=0.22
P=0.099
P=0.24
group 1
group 2
group 3
group 4
group 5
group 7

C

ConA stimulation induced less proliferation in groups 2 (wild type treated), 3 (low dose) and 5 (intermediate dose) compared to group 1 (untouched control); no difference was detected in groups 4 and 7 (low and high dose w/ RA; Figure 16B). At no time point in this study did we observe a proliferative response to insulin in these assays (Figure 16C).

In week 18, following 13 weeks of oral gavage, diabetic mice (one each from groups 1, 3, 4 and 5) were euthanized for analysis. Splenocytes were magnetically separated and used for suppression assay. CD4+CD25- cells from three of the four mice did not proliferate in response to stimulation with α-CD3ε and α-CD28. Figure 17 shows result of suppression assay for cells from mouse 4C1.

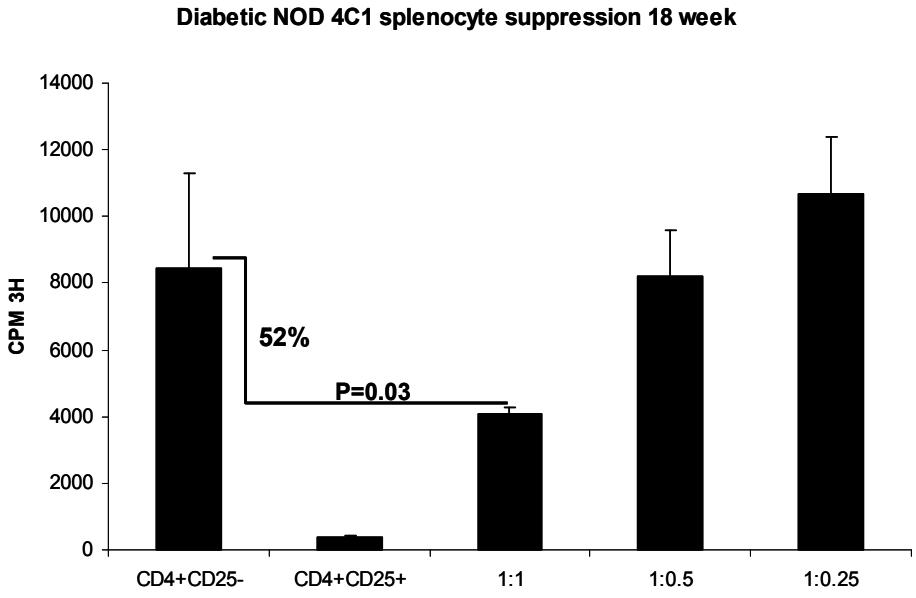
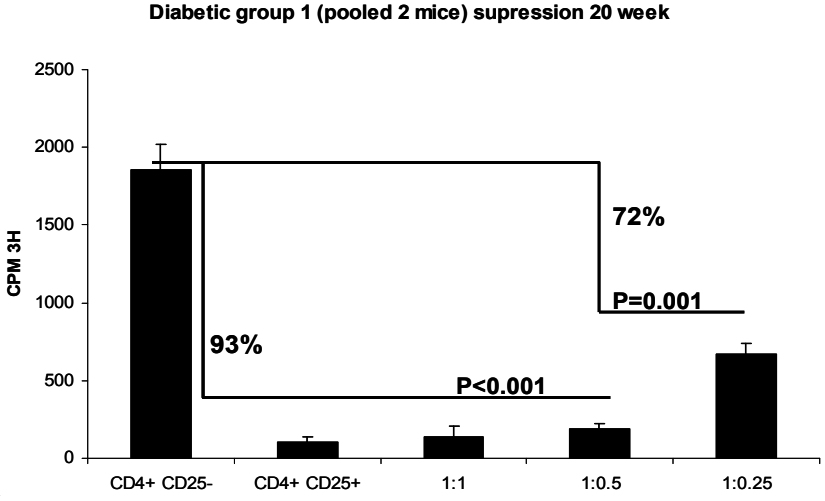


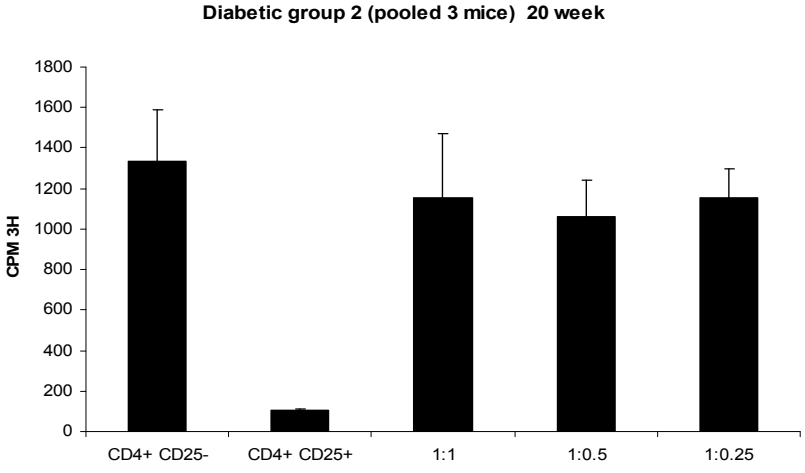
Figure 17: Suppressive capacity of Treg isolated from diabetic NOD splenocytes. 5×10^4 Teff co-cultured with decreasing proportion of autologous Treg in the presence of α-CD3ε and α-CD28.

When co-cultured with autologous CD4+CD25+ Treg in one to one ratio proliferation of the responding cells (Teff) was inhibited by 52%. This effect was not seen when the proportion of Treg was reduced to half.

Suppression assays were conducted with splenocytes isolated from diabetic animals sacrificed in week 20. For control groups 1 (untreated) and 2 (wild type treated) results are shown in figure 18.



A



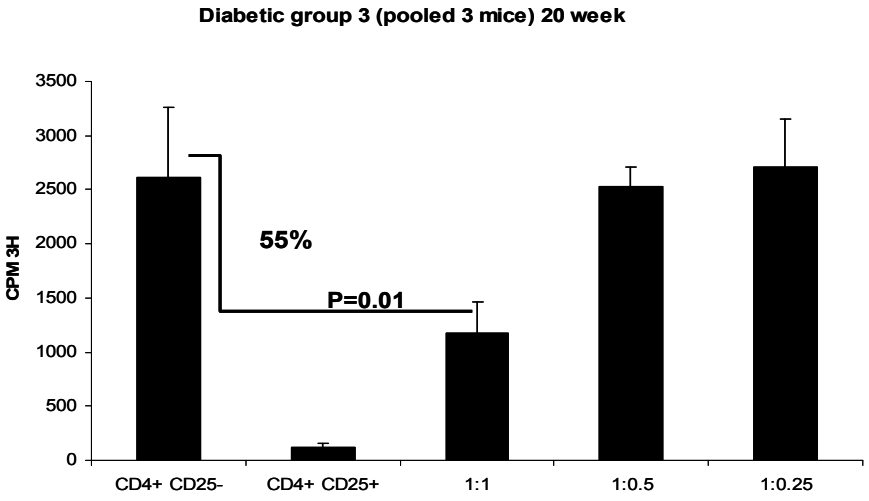
B

Figure 18: Variable results in control group suppression assays – Week 20.
5 x 10^4 Teff pooled within groups were co-cultured with decreasing proportion of Treg in the presence of α-CD3ε and α-CD28. A) Untreated control. B) wild type treated.

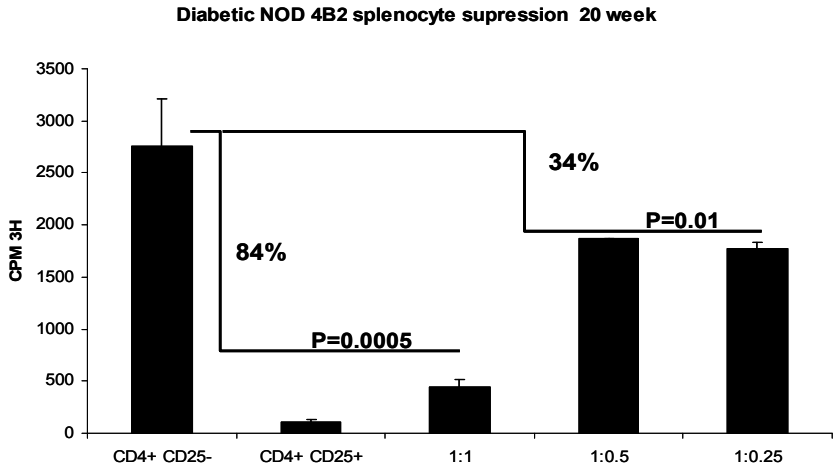
Treg cells from pooled group 1 splenocytes were highly effective at suppressing proliferation in the Teff population with 93% inhibition at one to one and one to half ratios. When Treg proportion was reduced to one quarter of the responding population marked suppression was still observed with a 72% reduction in proliferation (Figure 19A). This effect was not observed with pooled group 2 cells. There was no significant suppression under any co-culture condition in this assay (Figure 19B). Purified Treg cells from pooled splenocytes of three group 3 mice were able to suppress expansion of Teff by 55% when co-cultured at a one to one ratio. This effect was lost at lower Treg ratios (Figure 19A). A single mouse (4B2) was analyzed from group 4. Treg were effective at controlling proliferation of Teff cells demonstrating an inhibition of 84% at one to one, and 34% when Treg numbers were reduced relative to Teff (Figure 19B). The level of 3H incorporation in the responding population was not high in these assays. In proliferation assays, unsorted splenic lymphocytes activated with α-CD3ε and α-CD28 incorporated anywhere from 2.6-12 fold more ^{3}H (CPM). Nonetheless, ^{3}H incorporation in Teff population in suppression assays was more than 10 times greater than that seen in proliferation assay for unstimulated condition (Figure 19C) despite the fact that the cultures were seeded with 4 fold fewer cells as the starting population (5 $x10^{4}$ versus 2 x 10^{5}).

In week 23, splenocytes from three group 6 mice were pooled for suppression assay. A single individual from group 7 was also analyzed. There was no significant suppression of Teff proliferation by group 6 Treg (Figure 20A).

A



B



C

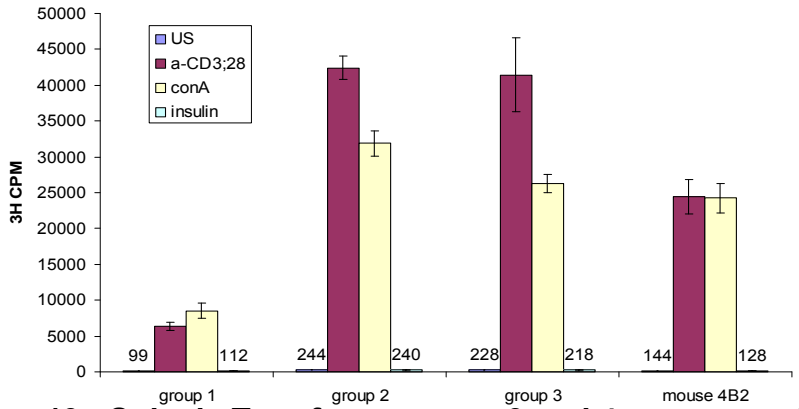
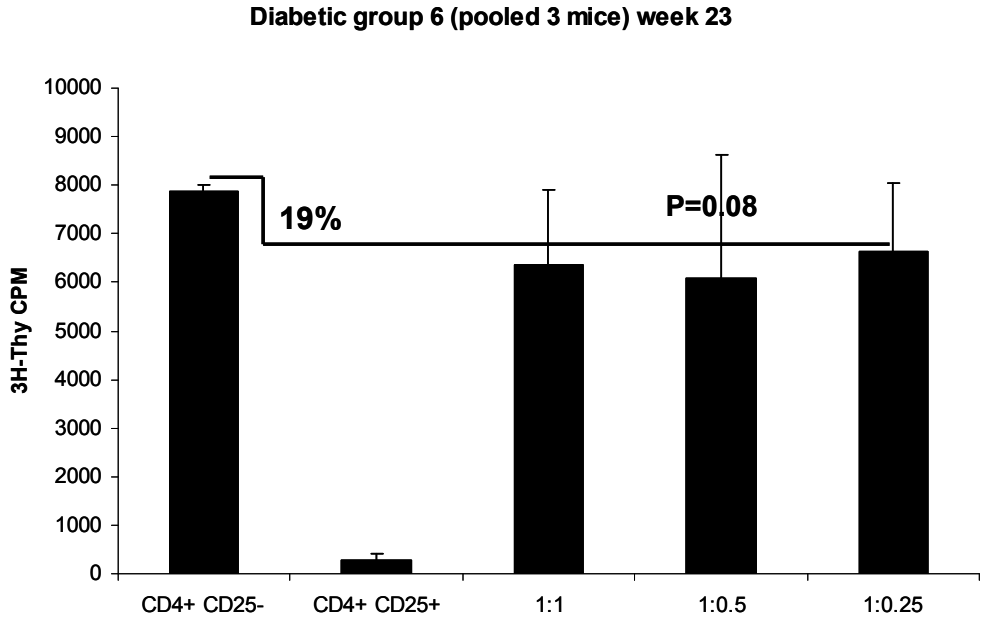
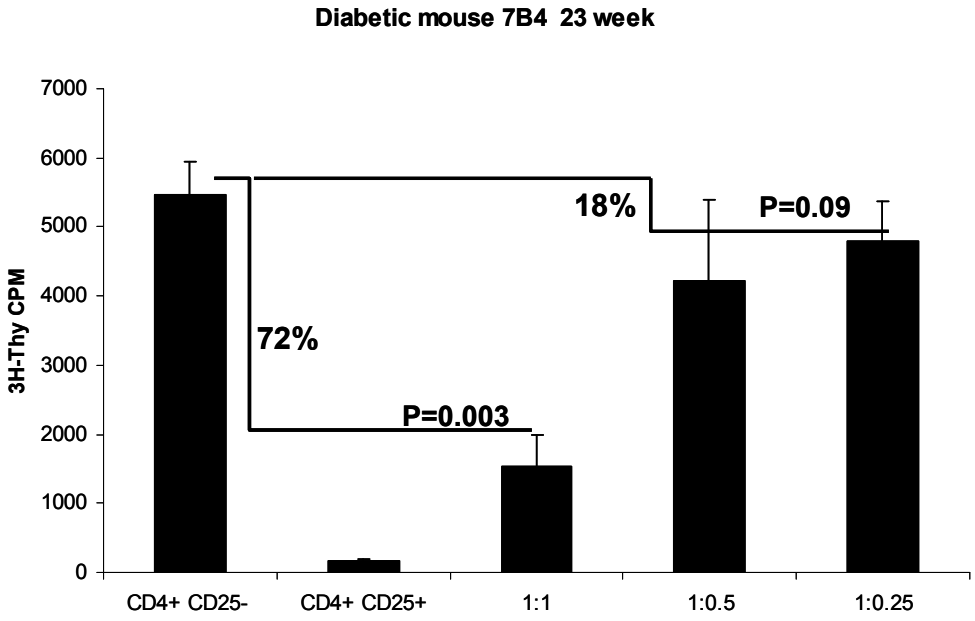


Figure 19: Splenic Treg from groups 3 and 4 suppressed Teff proliferation
Suppression assays A) group 3; B) group 4 individual. C) Proliferation assay using pooled or individual splenocytes.



A



B

Figure 20: Splenic Treg from a group 7 mouse suppressed Teff proliferation – Week 23.

Pooled group 3 splenic Treg were cocultured with Teff (A). Splenic Treg from mouse 1B4 cocultured with autologous Teff (B)

Treg purified from mouse 7B4 were able to suppress proliferation of autologous Teff by 72% at the one to one ratio. At lower Treg ratio, however this inhibition was lost (Figure 20B). The difference in suppression at the one to one ratio condition was significant between group 6 and mouse 7B4 (P=0.003).

By week 26, Treg isolated from pooled splenocytes of three group 7 mice, like the group 6 individual and the pooled group 1 Treg analyzed, did not demonstrate any suppressive function in this assay (Figure 21).

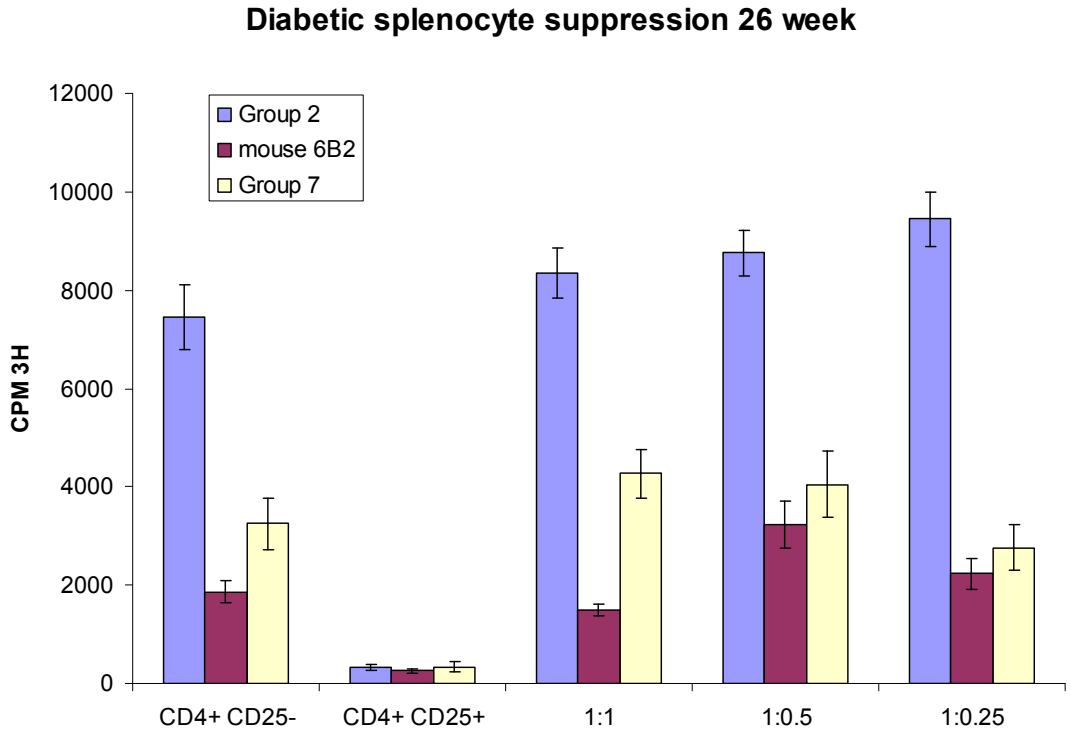


Figure 21: The suppressive capacity of splenic Treg was no longer observed by week 26.
Isolated Treg from spleens of mice sacrificed in week 26 were analyzed for suppressive capacity in co-culture with Teff cells.

Discussion

The potential to produce human therapeutic and antigenic proteins in transgenic plant systems has been developed to the point where preclinical studies utilizing animal models must be employed to evaluate the safety and efficacy of these products. From an environmental and food safety perspective plastid transformation technology offers some distinct advantages over nuclear transgenic systems particularly in transgene containment. To orally deliver bioencapsulated pharmaceutical proteins requires the production of transgenic food crops. The risk of transgene escape and genetic contamination of commodities headed for human consumption demands the highest level of assurance to safety. Additionally, to move beyond model systems and begin to address human suffering the level of foreign protein accumulation must reach biological significance.

In studies aimed at induction of oral tolerance by feeding transgenic plant material, CTB-Pins (Arakawa et al., 1998) and CTB-InsB_3 (Li et al., 2006) expressed in nuclear transgenic plants accumulated up to 0.1% in *S. tuberosum* tubers and *N. tabacum* leaves respectively. Arakawa et al. (1998) were able to demonstrate suppression of insulitis and delayed onset of T1D in NOD mice fed raw tubers, however this level of expression would preclude translation of the dosing format to human clinical trials. Our revised estimate of CTB-Pins accumulation in transplastomic *L. sativa* indicates that up to 300 fold more CTB-Pins is present in mature leaves (~2 mg g^{-1}) than was found in the potatoes used in the 1998 study.

Accumulation of foreign protein in plastids has to be evaluated on a case-by-case basis with regard to both the protein and the plant host in which is expressed. As we have seen with CTB-Pins in transplastomic *N. tabacum* and *L. sativa* lines, proteins predicted to be fully soluble, when present in large quantity may form aggregates, associate with membranes or participate in other unexpected interactions resulting in up to 90% of the target protein being excluded from the soluble fraction. That the majority of CTB-Pins was bound in the pellet material from centrifuged plant extracts leads us to speculate about the implications of this finding for calculating doses. Following digestion by gut acids and intestinal flora the amount of autoantigen available to participate in lymphoid interaction in the GALT is, at this time, unknown. This uncertainty is further compounded by the limited information available regarding the quantity of antigen that can be effectively detected by gut luminal sensing mechanisms and/or endocytosed *via* GM_1 ligation. These uncertainties are not limited to our study and may be reflected in variable results in oral tolerance approaches (Strobel, 2001; Faria and Weiner, 2005). Nevertheless, we are reporting the dose regimen in this study based on the total CTB-Pins found in leaf tissue homogenates, as this is the material we delivered to the mice.

Over the course of 30 weeks, we monitored blood glucose levels in our NOD colony to determine if our oral gavage treatment was affecting the onset of diabetes. The two most notable outcomes were seen in group 4 (low dose w/ RA) and group 7 (high dose w/ RA). In week 13 we observed the first cases of diabetes in the colony, while group 7 mice were diabetes free until week 22. Although the first cases of

diabetes were diagnosed early in group 4, at week 30 this group had the lowest incidence of onset overall. While these two groups received identical doses of RA, group 4 mice received approximately 5-fold lower dose of L100 leaf material per week suggesting the differential effect of these treatments resulted from the variable administration of CTB-Pins.

It has been reported that there are generally two primary routes through which oral tolerance may be established: low dose induction of Treg-mediated active suppression and high dose induction of clonal anergy and/or activation induced deletion of autoreactive Teff (Faria and Weiner, 2005). This description is likely an oversimplification and these broadly defined mechanisms may overlap, yet the different types of protection we saw in these high and low dose groups was suggestive. One attractive speculation could be drawn from the phenomenon of bystander suppression, shown to be associated with low-dose induced active suppression by Treg (von Herrath, 1997; Faria and Weiner, 2005). Bystander suppression occurs when Treg specific for a particular antigen functionally suppress Teff specific for some other antigen(s), provided that the 'bystander' antigen(s) is found within the same tissue. In the case of T1D numerous ß cell autoantigens have been described (Atkinson and Maclaren, 1994; Tisch and McDevitt, 1996; Tian et al., 1997). High dose driven anergy and/or deletion of Teff with specificity for proinsulin epitopes could provide the early protection we observed in group 7. Ultimately, as the destructive potential Teff with other ß-cell specificities was realized, the protective effect was overcome. In contrast group 4, although after some delay, experienced a more durable protection that could have

resulted from Treg mediated suppression of the entire autoreactive Teff complement that participates in the pathology of T1D in the pancreatic islets.

Under the experimental conditions in this study we did not observe long term protection in the treatment groups that received CTB-Pins *L. sativa* but no RA supplement. Previous work from our lab demonstrated that similar feeding of *N. tabacum* expressing CTB-Pins to NOD mice lead to amelioration of insulitis (Ruhlman et al., 2007). Insulitis was evaluated in the previous study at 12 weeks of age following 7 weeks of oral delivery and the study was terminated. It appears from our current findings that including an RA supplement delayed and decreased the onset of diabetes (hyperglycemia) in NOD mice compared to those that received antigen alone. Several studies suggest that RA produced by gut-localized DC plays an integral role in the establishment of tolerance to antigens presented via the oral route by driving the conversion of naïve T cells into $FoxP3^+$ Treg (Benson et al., 2007; Mucida et al., 2007; Sun et al., 2007). Furthermore it has been demonstrated that feeding NOD mice with all trans RA (Van et al., 2009), a synthetic analog of RA (Miwako and Shudo, 2009), or polyphenol-rich grape powder and/or an RA supplement (Zunino et al., 2007) can significantly reduce insulitis and hyperglycemia. In terms of longer term protection against the onset of fulminant diabetes, we are left with a difficult question: what was the contribution of CTB-Pins feeding to the observed protection in groups that received RA supplements? This question will, for now, remain open as we did not include a relevant control, NOD mice administered RA without antigen.

We have evaluated the capacity of splenic Treg to suppress proliferation of Teff in assays that included cells from single animals or cells pooled from several animals with groups. There are several challenges in deriving pertinent information from the results of these analyses. All of the cells utilized in these assays were isolated from animals that were overtly diabetic, the majority having been diagnosed 5 to 6 weeks prior to sacrifice and nearly all with blood glucose levels in excess of 599 mg dL^{-1}. In the case where suppression is observed it seems unlikely that this phenomenon is related to protection from disease given that these animals were, in fact, not protected but rather were quite ill at the time of analysis. Further confounding any comparison was the use of both single mouse analysis and pooled splenocytes for *in vitro* suppression studies. For example, in week 23, four diabetic mice were sacrificed. Group 6 was represented by three animals whose cells were pooled for analysis. Among the group 6 mice were two individuals that had been diagnosed for two weeks with blood glucose greater than 599 mg dL^{-1} and one that had been diagnosed 8 weeks prior and weighed just 15 grams at the time of sacrifice. In contrast a single group 7 mouse was analyzed at week 23 that had been diagnosed one week earlier with blood glucose of 476 mg dL^{-1} at the time of sacrifice.

With regard to Treg suppressive capacity as a function of the diabetic status of mice, several groups have demonstrated that there is no apparent difference in the frequency or performance of isolated NOD Treg as a result of disease onset, overt diabetes or advance in age, at least up to 16 weeks old (Mellanby et al., 2007; D'Alise et al., 2008). These studies have implicated Teff cells in the breakdown of tolerance to

self antigens in that they are resistant to suppression by Treg. Using cells isolated from NOD mice (D'Alise et al., 2008) and diabetic human peripheral blood mononuclear cells (Schneider et al., 2008) it has been shown that these Treg were fully capable to suppress Teff proliferation when the Teff population was isolated from a non-diabetic source, whereas the Teff cells from diabetic subjects or mice were insusceptible to the inhibition by control Treg.

In our study all suppression assays were carried out at 18 weeks and beyond. Additionally, splenocytes were separated based on antibody specificities for surface proteins with no regard to antigen specificity and stimulated with pan-activators α-CD3 and α-CD28, therefore these assays in no way assess the functional state of antigen specific Treg. Furthermore NOD mice have documented susceptibility to several autoimmune disorders suggesting another potential limitation in the usefulness of these suppression assays to evaluate the participation of Treg in T1D specifically (Rivero et al., 1998). Considering the advanced disease state of these animals it is likely that other complications of T1D were also confounding results.

Although the onset of diabetes in group 7 and 4 was significantly delayed and reduced, respectively, when compared to untouched control mice, the margin of difference at week 30 between group 4 and group 7 or the wild type treated group and groups treated with CTB-Pins without RA was reduced. Group 6 and 7 were treated twice per week, group 5 and 3 (intermediate and low dose; no RA) once per week and wild type treated (group 2) once per week. In addition to treatment, all animals, including the ‘untouched’ group 1, were screened twice weekly for hyperglycemia. We

speculated about the effect of handling on onset of diabetes. Elias (1994) reports that frequent handling can cause an elevation in the incidence of diabetes in NOD mice, likely due to stress (Elias, 1994). This argues against a handling effect with regard to the final outcome in our colony as groups with less frequent contact had a higher incidence of diabetes. At this time we cannot appropriately address what parameter caused this effect in our experimental setting.

Ultimately we have shown that oral delivery of bioencapsulated CTB-Pins in *L. sativa* leaf homogenates can delay and possibly prevent the onset of diabetes in NOD mice when RA supplement is provided compared to untouched mice. Whether delay or prevention is achieved appears to be a function of antigen dose suggesting that future approaches to affect prevention should consider early high dosing to facilitate delay and continued low dosing to sustain a durable response to treatment.

METABOLIC ENGINEERING OF A MODEL ANGIOSPERM TO ACCUMULATE SULFER AMINO ACIDS

Introduction

Approximately 65% of the global food supply is derived from plant sources and estimates exceed 80% among the least developed nations (Young and Pellett, 1994). The nutritional status of all animals, including humans, is directly or indirectly dependent on plants. As autotrophic organisms, plants have the capacity to acquire elemental compounds for the synthesis of complex macromolecules. In addition to their role as the center of photosynthetic energy production, plastids represent the metabolic powerhouse of plant cells. Nuclear encoded enzymes involved in the synthesis of starch, amino acids, fatty acids, vitamins and other intermediates and products of secondary metabolism are localized in the plastid (Ruhlman and Daniell 2007).

While plants synthesize all twenty proteinaceous amino acids, animals must consume histidine, arginine, phenylalanine, valine, threonine, tryptophan, isoleucine, lysine and methionine in the diet. Several others, including cysteine, cannot be synthesized *de novo*, but become available through metabolic activity on the essential amino acids (Harper, 1994). In livestock feed and forage and human diets where legume seeds and starchy root products comprise a large proportion of protein intake, deficiencies in the sulfur amino acids methionine and cysteine can have a significant impact on growth, productivity and maintenance of health. Ruminant animals, which are able to derive some of their essential amino acid requirement from the activity of gastric microorganisms, have demonstrated improved growth and productivity when their feed

is supplemented with methionine. In the US livestock producers utilize more than 50 thousand metric tons of methionine supplement each year, a practice which negatively impacts the environment and economic returns to the farm.

Biotechnological approaches have been investigated to improve the content of methionine and cysteine in plants. As animals are able to convert methionine to cysteine through their metabolism, strategies to enhance methionine accumulation have been utilized as a means to improve overall sulfur amino acid content in transgenic plants. Recombinant expression of sulfur-rich proteins such as the 2S abumin of *Helianthus annuus* (sunflower seed albumin; SSA) in seeds or leaves has been evaluated in several crops with mixed results (Tabe et al., 1995; Molvig et al., 1997; Christiansen et al., 2000; Hagan et al., 2003; Chiaiese et al., 2004). For example, SSA was found to comprise up to 7% of the extractable seed protein in *O. sativa* nuclear transgenic lines, yet total seed sulfur was unchanged in relation to parental lines (Hagan et al., 2003). Despite ample supply of exogenous sulfur to the experimental lines this group observed a redistribution of sulfur amino acid among the endogenous seed proteins to support synthesis of SSA. Several legume crops have been modified to express SSA including *Trifolium repens* and *Medicago sativa*. As these plants are employed predominantly for pasture forage, leaf expression was investigated revealing SSA accumulation up to 0.1% in leaf tissue (Tabe et al., 1995; Christiansen et al., 2000). This represents a significant enhancement and was predicted to be sufficient to impact wool yield and quality (Tabe et al., 1995). The prospect of releasing nuclear transformed pasture legumes raises serious concerns with regard to the introgression of

transgenes into non-target agricultural stands, particularly in the case of a seed albumin that is a demonstrated immunogen (Kelly and Hefle, 2000). *Trifolium repens* and *M. sativa* are pollinated by bees allowing for pollen-borne transmission of the SSA gene. The implications of unintentional SSA expression in *M. sativa*, a commonly utilized human food commodity, restrict the application of this technology to the field. Furthermore, in *Medicago* and *Trifolium* plastids are bi-parentally inherited, which limits the utility of plastid transformation for gene containment.

An alternative approach to establish transgenic plants with elevated levels of methionine has focused on metabolic engineering of the biosynthetic pathway. In plants methionine, along with threonine, isoleucine and lysine, is derived from aspartate by the consecutive action of several enzymes. The primary regulation on methionine synthesis appears to act at the level of the first pathway enzyme that is exclusive for its production, Cystathionine γ-Synthase (CGS; Bartlem et al., 2000).

Experimental strategies to favor CGS in the competition with Threonine Synthase (TS) for O-phosohomoserine (OPH), their mutual substrate, have led to enhanced concentrations of methionine and its derivatives in plant cells (Zeh et al., 2001; Kim et al., 2002). TS activity is stimulated by the presence of S-adenosyl-L-methionine (SAM; (Madison and Thompson, 1976; Curien et al., 1996) a metabolite of methionine that represents up to 80% of the methionine pool and the effectual end product of the methionine branch (Giovanelli et al., 1985). TS has as much as 250-fold higher affinity for OPH (Ravanel et al., 1995; Ravanel et al., 1998) and while a number of biotechnological interventions have resulted in modulation of methionine accumulation,

changes in threonine levels have been insignificant (Inaba et al., 1994; Chiba et al., 1999; Bartlem et al., 2000; Zeh et al., 2001; Kim et al., 2002). CGS is closely regulated at the level of translation by SAM concentration in the cytosol of *A. thaliana* (Chiba et al., 1999; Onouchi et al., 2004; 2005) and *S. lycopersicum* (Katz *et al.,* 2006) prior to its translocation to the plastid where its activity is found (Ravanel et al., 1998). SAM accumulates in the cytosol as the product SAM synthetase and is involved in a panoply of functions in addition to providing methyl groups for transmethylation reactions (Nikiforova et al., 2005). The role of SAM in CGS regulation was elucidated in the *Arabidopsis thaliana* mutant *mto1* (methionine overaccumlation; Inaba et al., 1994) which was found to maintain elevated levels of CGS mRNA, protein and enzyme activity in the presence of methionine and its downstream metabolite, SAM (Chiba et al., 1999; Ominato et al., 2002; Onouchi et al., 2004). The current model for translation arrest *via* the SAM/*mto1* interaction, as described by Onouchi et al. (2005), includes nascent CGS polypeptide elongation arrest within the ribosome followed by mRNA degradation initiated from the 5' end of the transcript.

The product of the CGS reaction, cystathionine, derives its sulfur moiety from cysteine. While the activity of CGS is localized exclusively in plastids, the enzymes involved in synthesis of cysteine, are present in mitochondria and the cytosol as well. The Cysteine Synthase Complex (CSC) is composed of two independently expressed proteins, each with several isoforms encoded in the nucleus. Serine Acetyltransferase (SAT) and O-acetylserine (thiol) Lyase (OAS-TL) perform the step wise acetylation of serine and ß-replacement of acetate with sulfide, respectively, to yield cysteine (Wirtz

and Hell, 2006). The level of SAT activity is dependent on association with OAS-TL within the CSC. The complex is formed from two trimers of SAT and two dimers of OAS-TL and is stabilized by high sulfide and low O-acetyl-L-serine (OAS; SAT product). Although this arrangement yields a 1.5:1 ratio in favor of SAT, it has been demonstrated that OAS-TL is far more abundant in all cellular compartments than SAT, up to 400 fold (Schmidt and Jager, 1992; Hofgen et al., 2001). In isolated spinach plastids, OAS-TL activity was estimated to be 345 times that of SAT (Ruffet et al., 1994). As OAS-TL is inactive in the CSC and its accumulation so greatly exceeds that of SAT suggests that a large proportion OAS-TL is always active. This observation points to SAT as the limiting activity for cystiene synthesis.

In the interest of obtaining plants that accumulate methionine and/or cystiene we have generated two lines of transplastomic *N. tabacum*. Using our established model system we have introduced the native *N. tabacum* nuclear encoded genes *cgs1* or *sat4* into the plastid genome. Complete compartmentalization of CGS expression within plastids, where free SAM is limited (Ravanel et al., 2004), should abrogate translational inhibition leading to enhanced enzyme activity and concomitant accumulation of methionine. *Nicotiana tabacum* SAT4 is predicted to be a mitochondrial-targeted isoform (Wirtz and Hell, 2003) that is insensitive to feedback inhibition by cysteine (Noji et al., 1998). Over expression of SAT4 could improve the stoichiometric potential for CSC formation in plastids leading to the production of cysteine for accumulation or as a substrate for the downstream CGS reaction leading to methionine.

Materials and Methods

Vector construction

The coding region for *N. tabacum* Serine Acetyltransferase 4 (SAT4) and Cystathionine γ-Synthase (CGS) were provided as cDNA in pCR®2.1-TOPO® plasmid (Invitrogen) in *E. coli* cultures. Single colonies were grown in Luria-Bertani broth (LB; 1% tryptone, 0.5% yeast extract 1% NaCl) with 50 µg mL^{-1} ampicillin and plasmids were isolated by alkaline lysis miniprep (Sambrook et al., 1989).

Amplification of 5' ends and 5' end modification employed 1 µL of Platinum® Pfx DNA polymerase and the buffer provided (Invitrogen). Reactions were assembled in 50 µL total volume and performed in a PTC-100 Peltier thermal cycler (Bio-Rad) and included 5 µL of 10X PCR reaction buffer, 0.2 µM dNTP, 0.5 µM each of forward and reverse primers (see Table 11 for sequence) and sterile distilled water. Amplification cycles were carried out as described earlier using annealing temperatures shown with primer sequences and extension temperature of 68 ^{o}C. To facilitate TA cloning into the pGEM®-T Easy plasmid (Promega Corporation, Madison, WI), 1 µL Taq polymerase was added to the reaction and extension was continued at 72 ^{o}C for 10 min. PCR products were examined by electrophoresis, excised with a sterile blade and purified using the QIAquick gel extraction Kit (Qiagen). Fragments were ligated into the multiple cloning site of pGEM®-T according to the manufacturer's instructions. Chemically competent XL1-Blue® *E. coli*. cells (Statagene) were incubated with 2 µL of the ligation reaction in 14 mL Falcon® tubes (Becton Dickinson, Franklin Lakes, NJ), on ice for 20

min. Cells were subjected to heat shock for 45 sec in a 42 ^{o}C water bath. After 2 min on ice, 950 µL SOC (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_{2,}$ 20 mM glucose) was added and reaction was incubated for 1 hour at 37 ^{o}C in a rotary shaker (~150 rpm). These cultures were distributed on LB solidified with 1.5% agar containing ampicillin (50 µg mL^{-1}), 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-gal; 80 µg mL^{-1}) and isopropyl-1-thio-β-Dgalactopyranoside (IPTG; 20 mM). Plates were incubated at 37 ^{o}C for 16 hours. White colonies were selected and grown in 5 mL LB with ampicillin for 16 hours for plasmid isolation. Recovered plasmids were digested with NdeI to determine the orientation of the inserts.

Table 11: Gene specific primers.

CGS-NdeI: AGTC**CATATG**GCTAAGGC	
CGS-3': AATGTGATGTTCTGGATGACTTTG	53 ^{o}C
SAT-NdeI: GCC**CATATG**TCCACGAAT	
SAT-3': AGCCAAGATTGAAGTTATG	54 ^{o}C

The 5' end of the original CGS coding region in pCR®2.1 was replaced with the PCR product with NdeI restriction site. Both plasmids were digested with HindIII and ApaI and the ~700 base pair 5' end fragment was swapped. Nucleotide sequence of recovered plasmids (pCR®2.1-NdeI-CGS) was confirmed. The plastid transformation vector pLD-CtV containing the T7 gene 10 translation control region was digested with NdeI and NotI as was pCR®2.1-NdeI-CGS. Ligation of the CGS coding region the transformation vector yielded the final plasmid pLD-CtV-g10-CGS.

The modified 5' end of SAT was digested with NdeI and NotI from the pGEM®-T plasmid and inserted into a pCR®2.1 plasmid that contained the *N. tabacum psbA* 5'

UTR. The UTR-5' NdeI-SAT fragment was transferred to pLD-CtV following EcoRI digestion. Finally the 3' SAT coding region was excised from the original pCR®2.1 plasmid by NruI and NotI digestion and the entire coding region was reassembled producing the plasmid pLD-CtV-5'UTR-SAT. This final plasmid was transformed into *E. coli* strain JM-39 (*cysE*$^-$; (Jones-Mortimer, 1968). Nucleotide sequence of recovered plasmid was confirmed.

Generation of *cis*-genic lines

Seeds of wild type *N. tabacum* cv TN90 (Lancaster Laboratories, Lancaster, PA) were surface sterilized in a 3% hypochlorite solution, rinsed three times in water and plated on MSO media solidified with 5.8 g L^{-1} Phytablend® (Caisson). Young, fully expanded leaves 4-5 cm^2 were placed abaxial side up on antibiotic free RMOP (MSO, 100 µg mL^{-1} myo-inositol, 1 µg mL^{-1} thiamine HCl, 1 µg mL^{-1} BAP, 0.1 µg mL^{-1} NAA) for bombardment using the PDS-1000/He Biolistic® device employing 1350 psi rupture disks and a target distance of 6 cm. The plastid transformation vector for generation of CGS or SAT lines was coated on to S04e gold particles (Seashell Technology, Ls Jolla CA) according to the manufacturer's instructions. Bombarded leaf samples were held in dark at 25 ^oC for two days prior to explant of 0.5 cm^2 pieces, abaxial side down onto RMOP media with 500 µg mL-^{1} spectinomycin dihydrochloride (Sigma). Primary regenerants were screened by PCR for the transplastomic event and positive shoots were subjected to an additional regeneration cycle on spectinomycin RMOP media. Following the second regeneration shoots were rooted in half strength MSO media with

500 µg mL^{-1} spectinomycin. Rooted cuttings were hardened in Jiffy-7® peat pellets (Jiffy Products) before transfer to the greenhouse.

Northern blot – CGS lines

Total RNA was extracted from wild type *N. tabacum* and CGS transplastomic lines using the QIAquick RNA extraction kit (Qiagen). Wild type and transplastomic samples, 10 and 5 µg respectively, were separated through denaturing agarose gels and northern blots were prepared and hybridized as described in chapter one. The full length *N. tabacum* CGS coding region (1350 bp) was utilized as the probe.

Confirmation of transgene integration, and determination of homoplasmy

Primary regenerants were screened by PCR to confirm transgene integration into the plastid genome. Primers 3P (5'AAAACCCGTCCTCEGTTCGGATTGC-3') and 3M (sequence in chapter 1) and 5P (5'-CTGTAGAAGTCACCATTGTTGTGC-3') and 2M (5'-TGACTGCCCACCTGAGAGCGGACA-3') were used to amplify sequence corresponding to the 5' and 3' ends of the integration cassette (Daniell et al., 2005). Total genomic DNA was isolated from rooted second regenerants and wild type plants for Southern blotting. Five micrograms of genomic DNA was digested with BglII (CGS) or AflIII (SAT), separated on agarose gels and transferred to nitrocellulose membranes as described above. Blots were hybridized with radiolabeled flanking sequence probes and exposed to autoradiography film as above.

CGS enzyme assay

CGS activity was measured as described by Ravanel et al. (1995). Mature source leaves of 8 week old, greenhouse grown plants were collected at 2:00 pm. Leaves were immediately frozen in liquid nitrogen and stored at -80 °C until the assay was performed. Frozen leaf tissue was ground in liquid nitrogen and 500 µL ice-cold extraction buffer was added (20 mm MOPS [3-(N-morpholino)-propanesulfonic acid]-NaOH pH 7.5, 2 mM dithiolthreitol, 100 mM pyridoxal 5′-phosphate (PLP), 0.1% (v/v) Triton X-100, 1 mM EDTA, and 0.2% (w/v) phenylmethylsulfonylfluoride). Leaf homogenates were transferred to microcentrifuge tubes and centrifuged at 14, 000 x *g* for 15 min at 4 °C. Supernatant was transferred to a new tube and centrifuged a second time. The supernatant was desalted using pre-equilibrated illustra™ NAP™ 5 columns (GE Healthcare). Total protein in desalted extracts was quantified using the Bio-Rad Protein Assay Reagent (Bio-Rad). CGS activity was measured in a 100 mL volume containing 20 mM MOPS-NaOH (pH 7.5), 2 mM dithiolthreitol, 0.1 mM PLP, 2 mM L-cysteine, 5 mM O-phospho-L-homoserine, and 0.2 mM aminoethoxyvinyl glycine hydrochloride (AVG). Assays were initiated by adding 100 µg protein extract. Reactions were incubated 30 min at 24 °C. The reaction was stopped by boiling for 5 min and analyzed for substrate (L-cysteine) depletion by direct spectrophotometry of reaction aliquots or product formation (L-cystathionine) by HPLC separation and spectrophotometry.

To determine substrate consumption the concentration of L-cysteine remaining in the reaction mixture was determined using a standard curve generated from serial

dilutions of purified L-cysteine (Sigma) in assay buffer. 50 µL aliquots from each sample and standard were boiled for 10 min in 200 µL ninhydrin solution (2.5% ninhydrin in 60% glacial acetic acid 40% HCl) and 100 µL glacial acetic acid. Reactions were cooled on ice and 650 µL absolute ethanol was added. Absorbances were obtained at 560 nm. Activity was defined as the amount (moles) of substrate utilized per minute per mg of total protein in the assay.

For determination of product concentration, 50 µL aliquots of enzyme assay samples were derivitized with 100 µL Phthalaldehyde `solution (40 mM Phthalaldehyde, 40 mM Borate, 10% ethanol, 0.2% 2-Mercaptoethanol) and analyzed by reversed phase HPLC along with serial dilutions of purified L-cystathionine (Sigma). Derivitized standards and samples were separated on a 4.6 X 150 mm C18 column attached to a Waters® Breeze™2 HPLC system (Waters, Milford, MA) attached to a UV/vis detector (340 nm). Eluent A was 85 mM sodium acetate and eluent B was 60% acetonitrile in water. Elution protocol was 0 to 8 min, 100% B; 8 to 12, linear gradient to 60% A. Retention time was ~ 4.5 min. Activity was defined as the amount of product (moles) produced per minute per mg of total protein in the assay.

In vitro bioassay

Seeds harvested from CGS primary transformants were surface sterilized and germinated on half strength MSO media with spectinomycin. Wild type TN90 seeds were germinated on antibiotic free media of the same composition. After 7-10 days on emergence of cotyledons, seedlings were transferred to half strength antibiotic free

MSO media containing 50 μM ethionine (Sigma), 60 μM DL-propargylglycine (PAG; Sigma) or PAG plus 250 μM homoserine (Sigma).

Amino acid analysis

Leaf samples were harvested from CGS transplastomic plants at different developmental stages and extracted for free amino acids according to Hacham et al. (2002). Samples were ground in liquid nitrogen with four volumes of water:chloroform:methanol (3:5:12). Homogenates were transferred to microcentrifuge tubes and centrifuged at 14, 000 x *g* for two min. Supernatants were transferred to a new tube and pellets were resuspended in water:chloroform:methanol and centrifuged as above. Supernatants were combined. Approximately 1.6 volumes of chloroform:water (1:3) was added and samples were centrifuged again. The upper, aqueous phase was transferred to new tubes and allowed to dry under a vacuum. In two to three hours a yellowish pellet remained. These samples were packed in dry ice and shipped to the Danforth Plant Science Center (St. Louis, MO) and injected into a AccQ-Tag™ Ultra UPLC® amino acid analyzer (Waters) for quantitation of amino acids.

SAT enzyme assay

SAT activity assay was adapted from Stiller et al. (2007). Activity was measured in extracts from fully expanded leaves of *in vitro* grown plants. Leaves were immediately frozen in liquid nitrogen and stored at -80 °C until the assay was performed. Frozen leaf tissue was ground in liquid nitrogen, transferred to a microcentrifuge tube and suspended in 10 volumes 0.1 M Tris-HCl pH 7.6 with 60 mM

PMSF. Samples were vortexed and centrifuged at 14, 000 x *g* for 10 min at 4 ^{o}C. Supernatants were assayed for total protein using the Bio-Rad Protein Assay Reagent (Bio-Rad). Up to 80 µg of total soluble protein was incubated in assay buffer (0.63 M Tris-HCl-7.6, 1.25 mM EDTA, 1.25 DTNB, 0.1 mM Acetyl-Co A, 1.0 mM Serine) in a cuvette and absorbance at 412 nm was measured over 15 min to monitor progression of the assay. SAT activity was defined as the amount (moles) of serine acetylated per minute per mg of protein in the assay.

Results

Vector construction

To facilitate cloning of the *N. tabacum cgs1* and *sat4* genes into the plastid transformation vector via in frame ligation to 5' regulatory elements a split cloning approach was employed using PCR modification of 5' ends. The NdeI restriction site was incorporated as it contains the translational start codon at the 3' end. For the CGS construct a restriction site located in the polylinker of the intermediate vector at the 5' end of the coding region was used in conjunction with a unique gene-internal restriction site to reconstitute the entire coding sequence with the 5' NdeI end modification. The cDNA restriction map for the *cgs1* coding region is shown in figure 22A. The position of the unique internal *Hind*III restriction site is highlighted. The final transformation vector pLD-CtV-g10-CGS used to generate CGS transplastomic *N. tabacum* is diagramed in figure 22B.

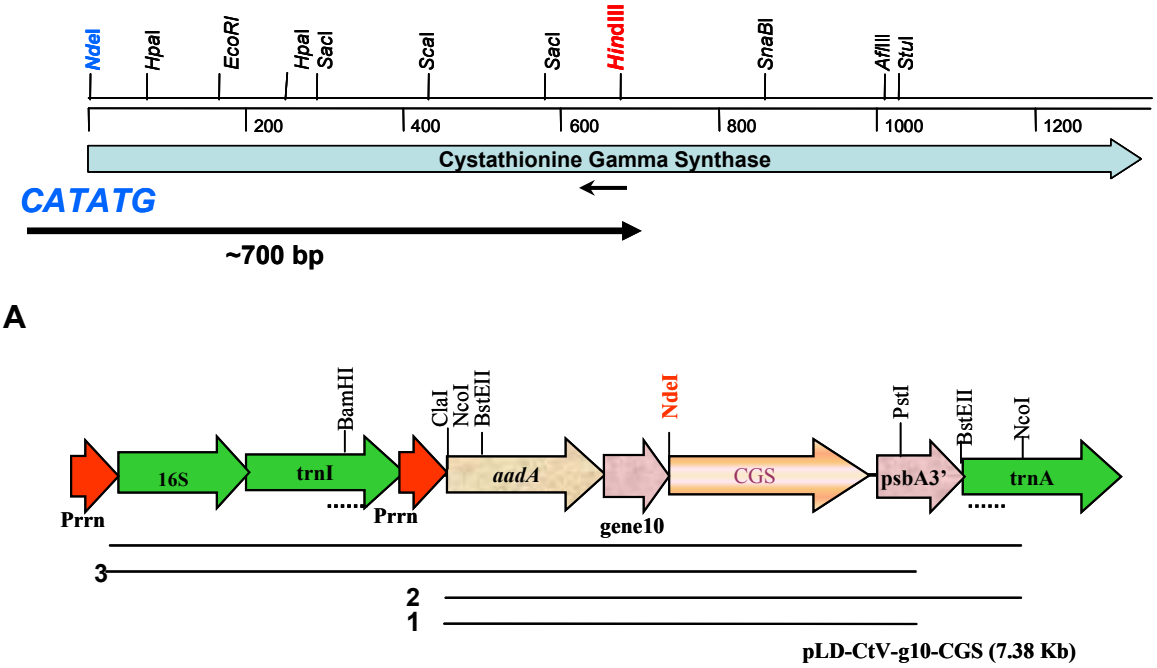


Figure 22: Schematic representation of CGS construct.
The cDNA encoding *N. tabacum* CGS is diagrammed with endonuclease restriction sites (A). Plastid transformation cassette and *N. tabacum* plastome flanking sequence are shown in (B). Broken lines indicate hybridization sites for Southern probes; numbered lines below indicate transcripts generated from the engineered and endogenous ribosomal operon promoter (P*rrn*).

The cDNA restriction map for the *sat4* coding region is shown in figure 23A. The position of the unique, internal NruI restriction site is indicated as are the NcoI and NdeI sites at the 3' end of the gene. The NcoI site, like NdeI, incorporates the 3' ATG start in its recognition sequence and could be used in cloning strategies were it not for its presence in the gene of interest. Due to the location of these sites within the *sat4* gene the modified 5' end was transferred to a pLD vector containing the *psbA* 5' UTR prior to reassembly of the coding region.

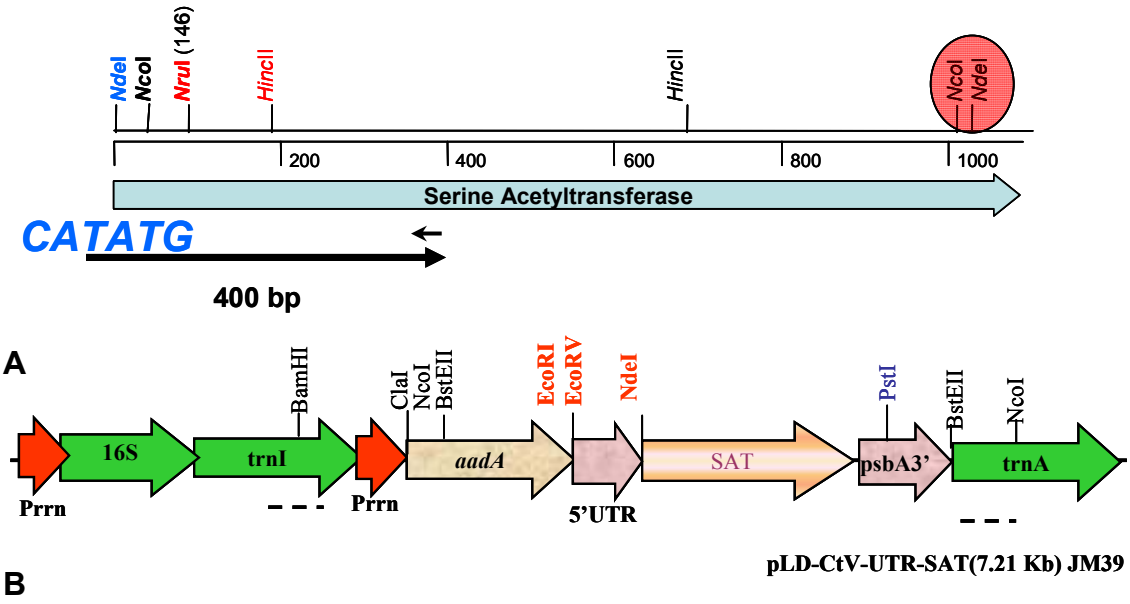


Figure 23: Schematic representation of SAT construct.
The cDNA encoding *N. tabacum* SAT4 is diagrammed with endonuclease restriction sites (A). Plastid transformation cassette and *N. tabacum* plastome flanking sequence are shown in (B). Broken lines indicate hybridization sites for Southern probes.

Generation and molecular analysis of *cis*-genic lines

Despite the use of numerous cell lines and media composition no positive clones were recovered for pLD-CtV-5'UTR-SAT. Although colonies grew on selective media following transformation with the final construct, restriction digests of isolated plasmids revealed that while spectinomycin resistance was intact, the region including SAT4 coding sequence was rearranged. When we transformed ligation reactions for the reassembled *sat4* gene in the pLD shuttle vector into the cysteine mutant *E. coli* strain JM39 we recovered the final construct pLD-CtV-5'UTR-SAT. The plastid transformation vector pLD-CtV-5'UTR-SAT is diagrammed in figure 23B.

For plastid transformation of *N. tabacum* cv TN90 we utilized S04e gold particles (Seashell Technology). The manufacturer describes this particle as a nanofabricated, gold-core particle with an average diameter of 550 nm. The product is provided in suspension and accompanied by binding and precipitation buffers and plasmid DNA was loaded onto particles according to the manufacturer's protocol. Our lab has routinely used Bio-Rad gold particles with a diameter of 600 nm employing both 1100 and 1350 psi rupture discs resulting in high efficiency transformation in *N. tabacum.* In consideration of the smaller particle size we chose to use 1350 psi rupture discs for these experiments, otherwise the transformation protocol by particle bombardment was carried out a previously described. Numerous regenerants were isolated from each intact leaf bombarded following 3-4 weeks on selective media. Primary regenerants were screened by PCR and positive shoots were explanted and subjected to an additional cycle of regeneration on spectinomycin media. CGS transplastomic lines were hardened and transferred to the greenhouse. These lines were indistinguishable from wild type plants and set abundant seed (Figure 24A).

A **B**

Figure 24: Phenotypes were different between CGS and SAT lines.
CGS lines (right) were indistinguishable from wild type (left) plants (A). *N. tabacum* transformed with SAT construct growing in vitro (B).

We were able to recover numerous primary regenerants from bombardment experiments using the SAT construct that were PCR positive using primers 3P and 3M (Figure 25A & B) indicating that the *aadA* gene had been integrated into the plastome between *trn*I and *trn*A as expected. PCR of several among these lines using the 5P and 2M primers (Figure 25C) resulted in amplification of the 2.68 kb expected fragment while several other PCR assays utilizing the 5P/2M primer combination did not yield a result.

We observed that there were different visibly deleterious phenotypes in some, but not all, SAT lines. In particular the line designated S5 was severely impaired, having unusually shaped, thickened and chlorotic leaves and shortened internodes (Figure 24B). This line could not be cultured out of sucrose-based media, developed lesions and never produced flowers or seed while several other lines, including PCR

positive lines S1 and S2 grew normally, were transferred to the greenhouse and were fertile.

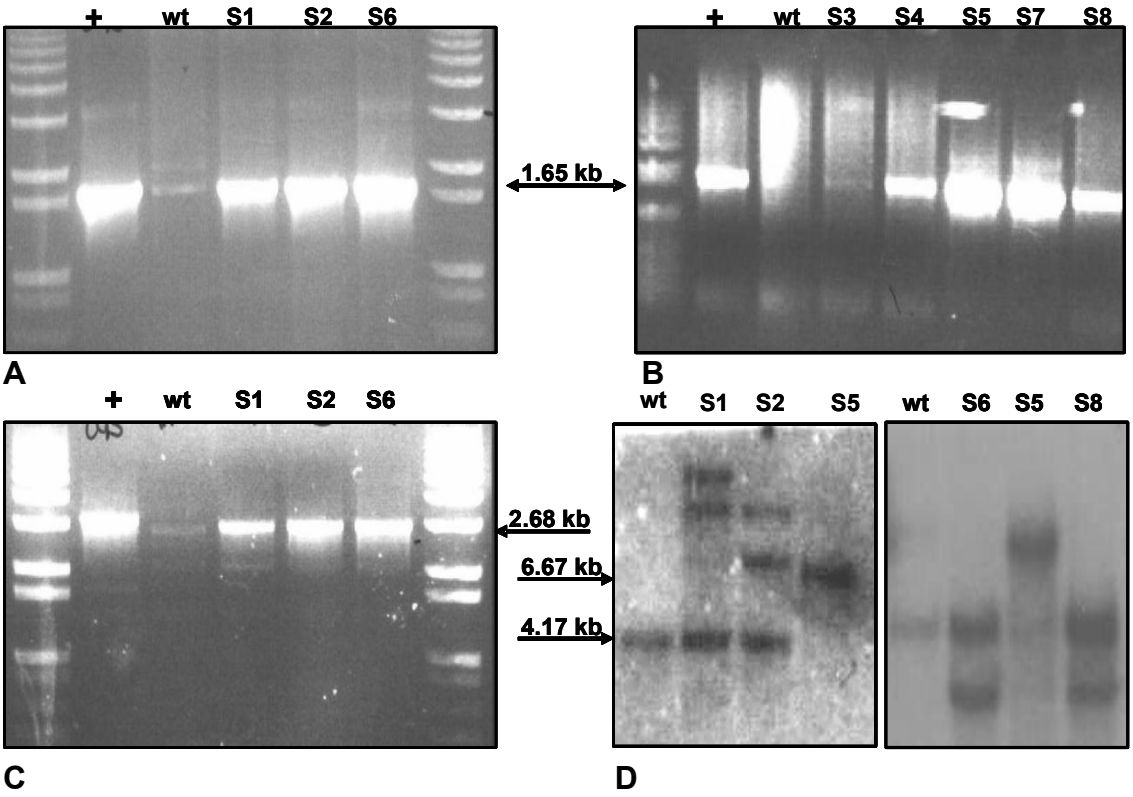


Figure 25: Confirmation of site-specific transgene integration and homoplasmy in SAT transplastomic lines.
A & B) 2P/3M products from SAT primary regenerants. C) 5P/3M products and D) Southern blot of total DNA from primary SAT regenerants. Oligonucleotides 3M and 5P hybridize within the selectable marker gene, 2P within the plastid trnI gene and 2M within the trnA gene (nucleotide sequence is given in text).

Line S5 was maintained though nodal propagation and repeated regeneration, up to 6 cycles, on spectinomycin media. Southern analysis performed on T_0 SAT lines demonstrated that 4 round regenerants of line S5 were homoplasmic for the transplastome whereas regenerants from lines S1, S2, S6 and S8 had a peculiar

hybridization pattern and a large proportion of wild type fragment following digestion with AflIII (Figure 25D).

Seeds collected from CGS transplastomic lines were harvested from greenhouse grown plants, sterilized and germinated on spectinomycin media. Genomic DNA was extracted and digested with BglII for Southern analysis. Blots hybridized with the radiolabeled flanking sequence probe revealed the expected 5.04 kb band and an absence of the wild type band indicating that these lines were homoplasmic (Figure 26A).

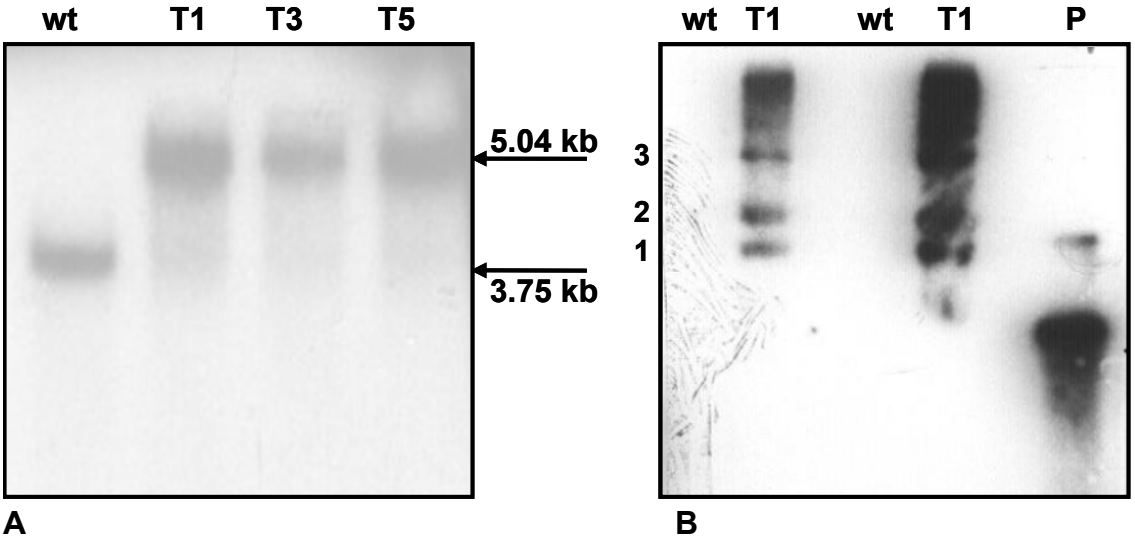


Figure 26: Confirmation of homoplasmy and CGS mRNA production in CGS transplastomic lines.
Genomic DNA from second generation CGS transformed lines was analyzed by Southern blotting following *Bgl*II digestion (A). Northern blot of CGS line T1 and wild type plants (B). Southern blots were probed with the native plastid genome flanking sequence; northern blots with the full length coding region for CGS1.

Northern blot of total RNA was conducted to confirm expression of the CGS transcript. Hybridization with the radiolabeled CGS coding region probe confirmed that

CGS mRNA was transcribed as a dicistron and, as expected for plastid transformants generated using the ribosomal operon as the integration site, larger forms of CGS transcript were detected (Figure 26B). We were unable to detect the endogenous, nuclear *cgs1* mRNA in transplastomic or wild type samples despite long exposure of blots to autoradiographic films.

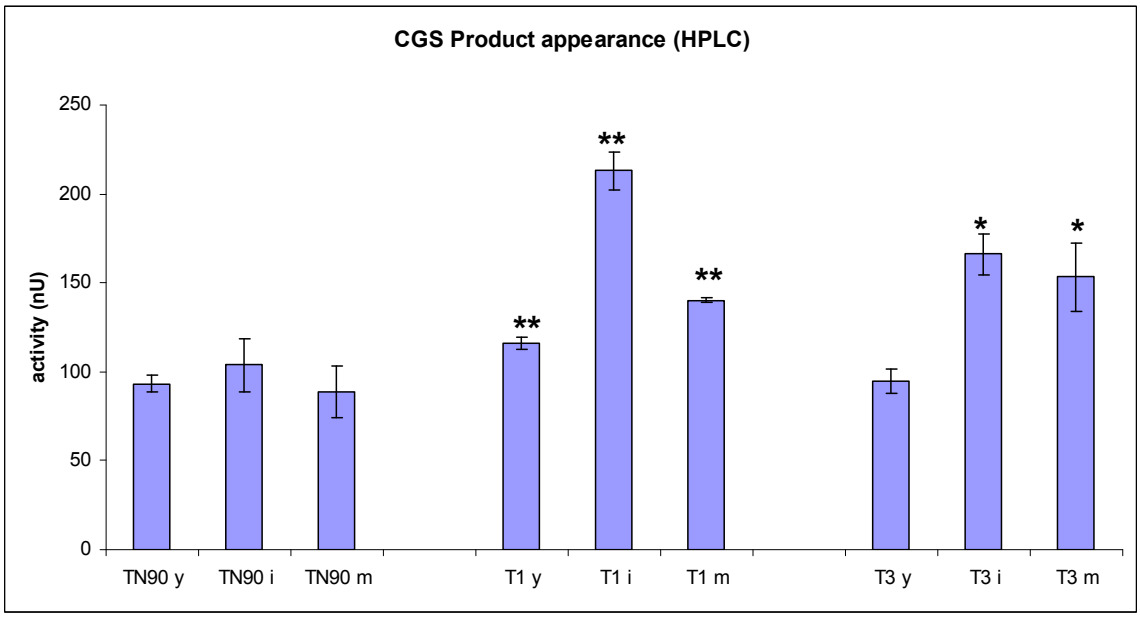
CGS enzyme assay

Extracts from young, intermediate and mature leaves of second generation CGS transplastomic lines were evaluated for enhanced enzyme activity by monitoring substrate consumption and product appearance. AVG was included in the reaction mixture to inhibit the activity of Cystathionine ß-lyase (CBL), the next enzyme in the methionine synthesis pathway.

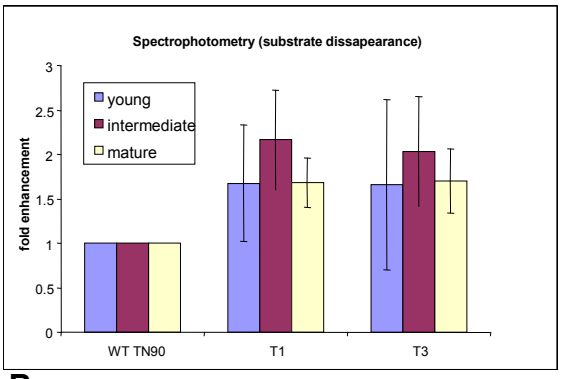
The reactions were separated by RP-HPLC and accumulation of CGS product cystathionine was determined by comparison to a standard curve. Activity was defined as nanomoles of cystathionine produced per minute per mg of total protein in the assay (nU; Figure 27A). In extracts from young, intermediate and mature leaves of transplastomic line T1, CGS activity was significantly elevated compared to wild type control extracts ($P<0.01$). While line T3 did not display enhanced activity in young leaf extracts, intermediate and mature samples were significantly elevated compared to control ($P<0.05$).

The results in figure 27B and C are presented as mean fold enhancement calculated from at least three independent tests. Utilization of substrate cysteine in leaf

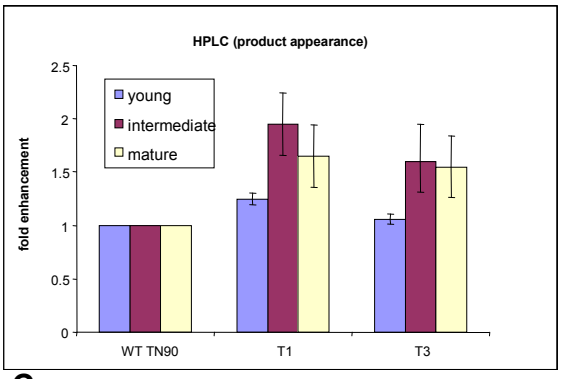
extracts was elevated in plastid transformants compared to wild type control, ranging from 1.6 to 2.4 fold. Accordingly product accumulation in plastid transformants ranged from 1.2 to 2 fold compared to controls.



A



B

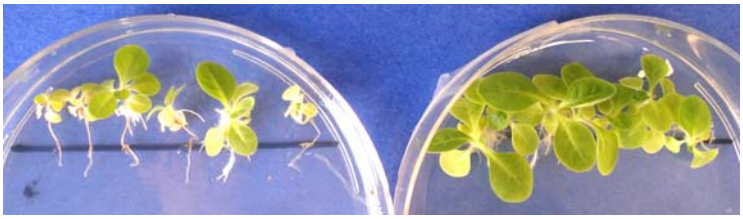


C

Figure 27: CGS activity was elevated in transplastomic lines.
CGS expression in plastid transformants elevated cystathionine production by leaf extracts. Data were analyzed by Student's t-test comparing activity in transplastomic leaves of different developmental stages to corresponding wild type leaves. Significant difference is shown by (*,**) indicating 95% and 99% confidence. CGS activity was defined as nanomoles of cystathionine produced per min per mg of total protein in the assay. Fold enhancement is reported based on mean activities from substrate consumption (B) and product appearance (C) assays calculated from at least 3 independent tests.

***In vitro* bioassay**

Seedlings from CGS transplastomic line T1 and wild type plants were grown on agar solidified MSO media containing PAG, which binds irreversibly to the CGS active site, or PAG with homoserine which can be converted *in planta* to provide the OPH substrate for CGS activity.



A

B

C

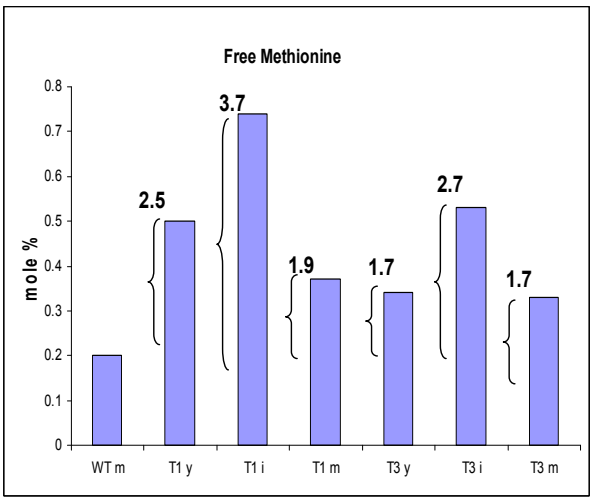
D

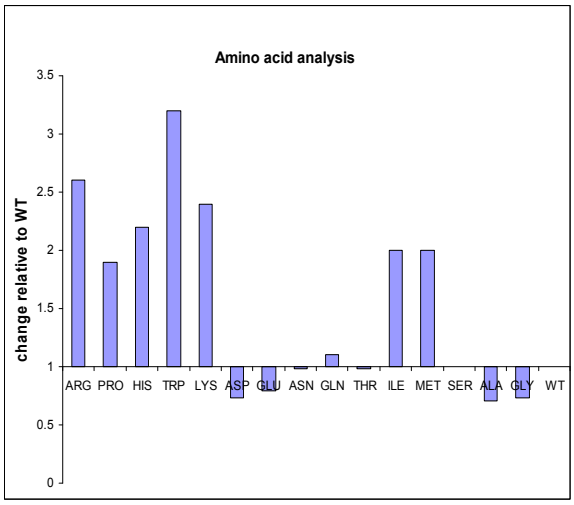
Figure 28: CGS seedlings showed partial resistance to ethionine and PAG.
Line T1 (A) and TN90 wild type (B) were grown on MSO media with 60 μM PAG alone (left) or with 250 mM homoserine (right). Line T1 (C) and wild type (D) were also grown on 50 μM ethionine.

Both wild type and transplastomic seedlings displayed impaired growth in response to PAG in the media, whereas only the transplastomic line appeared to recover with the provision of additional substrate (Figure 28 A and B). We also compared the effect of ethionine, a toxic methionine analogue, on the growth of transplastomic and wild type seedlings. While both sets of seedlings were impaired in root establishment on ethionine media, the CGS transformed line showed less retardation in terms of vegetative growth (Figure 28 C and D).

Amino acid analysis

To determine if overexpression of CGS in transplastomic lines had an effect on methionine accumulation in leaves we prepared leaf samples for injection into an amino acid analyzer. For methionine values are given in mole percent of total free amino acid in each sample. In keeping with the results from enzyme assay free methionine in mature leaves was elevated compared to wild type mature leaf control by 1.9 and 1.7 fold in transplastomic lines T1 and T3, respectively (Figure 29A). Free methionine accumulation seen in other developmental stage samples are comparable to variation seen in enzyme assays for the same stage leaf extracts.





A **B**

Figure 29: Accumulation of free amino acids differed between wild type transplastomic plants.

Samples were injected into an amino acid analyzer. In (A) values are given as mole % methionine of total amino acid in each sample. Brackets and numerical values represent the fold difference in mole % in different transplastomic samples compared to control sample. Changes in the free pools of several amino acids in mature leaves are shown as a ratio relative to wild type (B).

SAT enzyme assay

Leaf extracts from *N. tabacum* SAT transplastomic plants were assayed for elevated SAT enzyme activity in comparison to wild type controls. SAT activity was defined as the moles of serine acetylated per min per mg of total protein in the assay. Of the three lines investigated only line S5 had enhanced SAT activity in this assay, this difference was significant ($P<0.001$) and represented an 8.5 fold elevation. Extracts from the other two lines in this assay, S1 and S2, had significantly ($P< 0.01$ and $P<0.05$) lower SAT activity than the wild type (Figure 30).

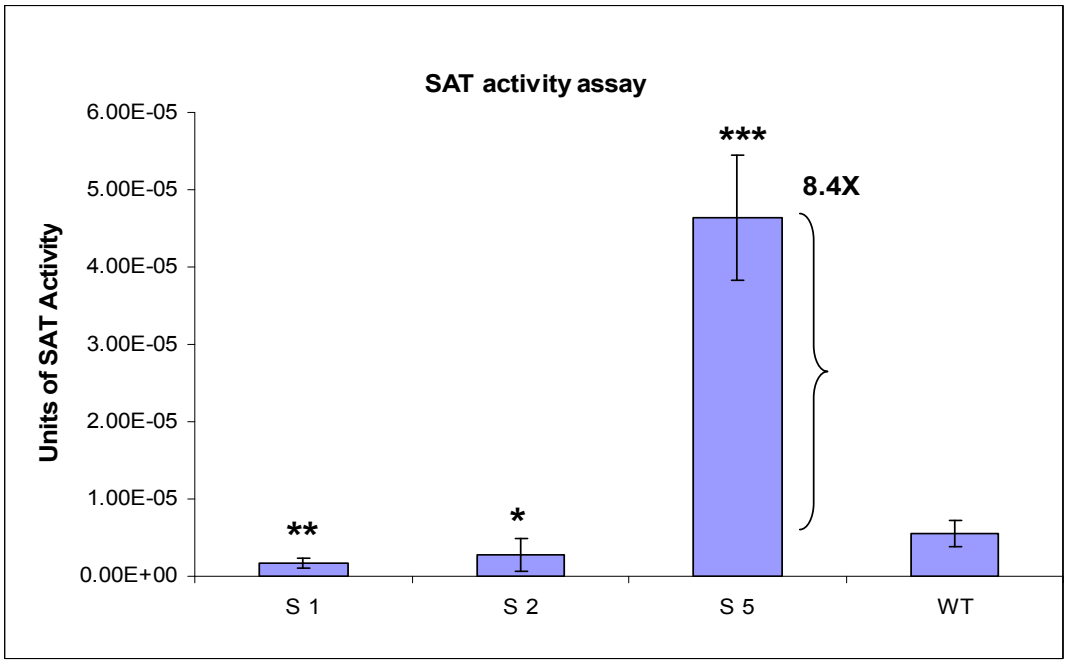


Figure 30: SAT activity was both positively and negatively affected in transplastomic *N. tabacum*.
Leaf extracts from *in vitro* grown transplastomic and wild type plants were analyzed for SAT activity. Units were defined as the moles of serine acetylated per min per mg of total protein in the assay. Significant difference from wild type is shown by (*,**,***) indicating 95%, 99% and 99.9% confidence.

Discussion

Both the quantity and quality of dietary intake influence productivity and health in animals. Many cultures, by choice or necessity, consume diets composed predominantly of plants which can lead to nutritional deficiencies even where adequate quantities are available. A number of staple commodities lack required levels of certain essential amino acids. In particular, legume crops are limited in the sulfur amino acids methionine and cysteine. As a protein constituent and having numerous metabolic roles methionine cannot be spared in the diet. The importance of adequate methionine

provision in early development is suggested by the involvement of its downstream metabolite, SAM, in methyltransferase reactions involved in epigenetic patterning (Rees, 2002; Brosnan and Brosnan, 2006). Several cytokine induced proteins including glutathione (cysteine) and metallothionein (methionine and cysteine) contain up to 50% sulfur amino acid and otherwise healthy post-surgery patients present with a reduced ratio of sulfur to nitrogen in the urine, suggesting sulfur amino acids are being retained in tissues (Grimble and Grimble, 1998; Grimble, 2006)

Supplementation of livestock feed with methionine and methionine analogues has been positively correlated with wool yield and quality in sheep, growth in lambs, swine and cattle; and weight gain, feathering and layer productivity in poultry (Amino Acids in Animal Nutrition, 2003).

Our homoplasmic lines expressed abundant CGS mRNA from the engineered and native ribosomal operon promoters. Northern blot of total RNA suggested that there was sufficient CGS mRNA present in transplastomic lines and that plastid expressed transcript was not subject to degradation (Figure 26B). We did not detect the native, nuclear CGS transcript in the wild type or transplastomic sample. If plastid transformants were accumulating methionine and SAM it could be expected that the endogenous transcript would be suppressed, nonetheless we would expect to detect the native transcript in wild type plant samples. As reported by Zhang et al (2001) the endogenous transcript encoding Anthranilate Synthase α-subunit (ASA2), a key enzyme in tryptophan synthesis, could not be detected by northern blotting and was barely detectable by reverse transcription PCR in transplastomic or wild type plants (Zhang et

al., 2001). Unlike ASA2, there is but a single copy of *cgs1* in the *N. tabacum* nuclear genome thus it may be that a more sensitive approach would detect the wild type transcript.

We evaluated CGS enzyme activity in crude extracts from leaves of various ages, harvested from mature plastid transformants raised in the greenhouse. Quantitative analyses of substrate consumption and product appearance were in agreement and demonstrated significantly enhanced CGS activity in transplastomic plants compared to wild type controls (Figure 27). While we did observe elevated CGS activity in these assays the level of transplastomic activity never exceeded three fold that of the wild type. Substrates were provided at a saturating level to avoid any potential limitation in these *in vitro* assays and a potent inhibitor (AVG) of the subsequent enzyme activity (CBL) was included in the reaction to preserve cystathionine accumulation. Corresponding to the level of enzyme activity in transplastomic lines was the level of free methionine in mature leaf extracts which were elevated approximately two fold over wild type (Figure 29). Further confirmation that our transplastomic *N. tabacum* lines were overexpressing CGS came from their partial resistance to ethionine and that they were able to utilize exogenously supplied homoserine to overcome PAG inhibition (Figure 28). In *A. thaliana* plants fed with 250 μM homoserine wild type plants did accumulate twice as much free methionine than unfed plants while the CGS overexpressors accumulated up to 15 fold more free methionine when fed with homoserine over transgenic plants grown on standard potting mix with N-P-K fertilizer (Kim et al., 2002; Lee et al., 2005).

We considered some factors that could affect translation of foreign mRNA in plastids. The composition of nuclear genes and genomes varies from plastids in terms of AT and GC ratio, with plastid genomes being more AT rich. This difference can affect translation efficiency through codon bias, i.e. the availability of tRNAs with the appropriate anticodon for the next amino acid in a growing polypeptide (Morton, 1993). Sequence analysis of the *cgs1* cDNA revealed that the unmodified nuclear-derived transcript is 54.8% AT overall with 45.3%, 60.1% and 49.2% A or T in the first, second or third position in codons. The anticodon specificity for plastid encoded tRNAs was found for 167 of 446 (37%) residues in the amino acid sequence. All other codons have corresponding first and second position matches allowing for wobble pairing and elongation of the CGS protein. This suggests that codon compositional bias would not likely be relevant deterrent of CGS expression in plastids. Furthermore, Daniell et al have recently demonstrated that, at least for some genes, plastid expression of foreign genes may be at an optimum and will not accumulate protein to higher levels as a result of codon optimization (Daniell et al., 2009). The abundance of the transplastomic mRNA pool may not provide a reliable predictor of foreign protein accumulation. Within the 5' end of the coding region of the *A. thaliana* CGS transcript Hacham *et al.* (2006) predict a stable secondary stem loop structure. Given the involvement of these types of structures in processing of plastid mRNA, trans-acting factor binding and ribosome association it is possible that this feature could interfere with efficient translation of the transcript in plastids (Alexander et al., 1998; Fedoroff, 2002; Merhige et al., 2005).

In keeping with previous reports our findings suggest that achieving overexpression of CGS enzyme activity may be insufficient to produce plants that hyper accumulate free methionine, that other factors may play an integral role in this regard. Like our transplastomic plants, the *A. thaliana* transgenic lines with limited SAMS activity had about three times more activity for CGS (Kim et al., 2002). The increase in CGS activity was likely due to the reduction in SAM content in the cytosol allowing for a higher level of translation of the CGS enzyme. Only about 20% of the *de novo* synthesized methionine in plant cells is incorporated into proteins, the rest is rapidly converted to SAM by SAMS (Giovanelli et al., 1985; Ranocha et al., 2001). In the absence SAMS conversion and without SAM-mediated regulation on CGS expression these plants accumulated massive quantities of methionine. The reality of these examples and others is that dramatic alteration of methionine metabolic pathways by a number of approaches, though resulting in abundant free methionine levels, often leads to severe deleterious phenotypes that would preclude any field application (Boerjan et al., 1994; Goto et al., 2002; Dancs et al., 2008). Ultimately our transplastomic plants accumulated up to 2 fold more methionine in leaves which could feasibly represent a significant enhancement in terms of yield and quality in livestock.

In an alternate approach, we sought to enhance SAT enzyme activity by expressing the feedback-insensitive *N. tabacum* SAT4 via plastid transformation. Attempts to recover homoplasmic *N. tabacum* plastid transformants yielded a single line, S5, which had severe phenotypic abnormalities and could not be cultured outside of an *in vitro* setting. We maintained this line through repeated regenerations and nodal

propagation as the clones always senesced prior to the development of reproductive structures. Several other spectinomycin-resistant regenerants were screened by PCR and Southern analyses (Figure 25). Enzyme assays and Southern analysis were conducted following a second round of selective regeneration and rooting of plantlets. Initial results from enzyme assays indicated marked differences in activity among second regenerants. Line S5 was clearly superior to all the other lines assayed for activity and Southern blots confirmed this line was homoplasmic (Figure 25D). Although primary transformants for several of the other regenerated lines gave expected results in PCR screening, a peculiar hybridization pattern was seen in Southern blots of genomic DNA isolated from these samples. Focusing on two lines, S1 and S2, enzyme assays were conducted to evaluate SAT activity compared to line S5 and wild type controls. These assays revealed that extracts from S1 and S2 lines had significantly less SAT activity than the wild type ($P<0.05$) while S5 extracts were significantly enhanced in SAT activity ($P<0.001$; Figure30).

During the construction of the vector for SAT we routinely recovered spectinomycin-resistant *E. coli* colonies that had unpredictable patterns when digested with restriction enzymes. It was not until we acquired the cysteine mutant strain JM39 that we were able to recover the pLD-CtV-5'UTR-SAT shuttle vector. Due to the prokaryotic nature of the plastid expression system, our final shuttle vector, containing plastid promoters and UTRs, is expressed in *E. coli* cells. We did not detect any abnormalities or sequence variation when the cells harbored the SAT sequence without a UTR to facilitate translation, nor were there any issues when just a portion of SAT was

present in pLD along with the UTR element. Accumulation of cysteine and its metabolites is known to be toxic in *E. coli* resulting in growth inhibition and oxidative damage to DNA (Harris et al., 1983; Park and Imlay, 2003).

Recent reports indicate that plastid localized CSC contributes little to the synthesis of cysteine in *A. thaliana* cells (Heeg et al., 2008; Watanabe et al., 2008; Krueger et al., 2009). Complete suppression of the plastid localized SAT isoform had no effect on total cysteine concentration in T-DNA insertion lines that maintained cysteine levels in the cytosol that exceeded plastid levels by more than 30 fold (Krueger et al., 2009).

Conditions of high sulfide and low OAS promote SAT and OAS-TL association within the CSC activating OAS synthesis. These conditions are met and the substrates for OAS synthesis, serine (Ho and Saito, 2001) and acetyl-CoA (Nikolau et al., 2000; Mentzen et al., 2008) are present in wild type plastids. By introducing mitochondrial SAT4 activity in the plastid compartment we may have concomitantly introduced the potential for various metabolic and signaling perturbations. Enzyme assay results for our phenotypically unstable line S5 indicate that we were able to influence SAT activity *via* plastid transformation. If, like in *A. thaliana*, only a minute fraction of total SAT activity can be attributed to *N. tabacum* plastids, the more than 8 fold elevation we observed in total extract activity from transplastomic line S5 suggests the level of active SAT enzyme in plastids is greatly enhanced. The severe deleterious phenotype in S5 could derive from undetermined physiological issues including reduction of the acetyl-CoA pool or defective redox sensing activities in plastids.

Our experience in the cloning stages to generate SAT transformation plasmids can be rationalized in the context of cysteine toxicity in *E. coli*. That plasmid rearrangement issues were resolved by using the cysteine auxotroph JM39 cells for cloning SAT with functional promoters and expression elements in *E. coli* further supports the notion of genomic toxicity. For our transplastomic *N. tabacum* lines S1 and S2 genomic toxicity could be driving rearrangement around the insertion site, a phenomenon suggested by the maintenance of a high proportion of wild type plastome copies and peculiar Southern hybridization patterns in these lines. It is not surprising that these lines maintain wild type genomes; rearrangement within the ribosomal operon, the site of SAT insertion, would deter progression to homoplasmy even under strong selective pressure (Svab and Maliga, 1993). These lines could eventually resolve back to the homoplasmic state, a process of gene conversion which occurs in plastids to resolve deleterious mutations (Khakhlova and Bock, 2006).

As a center of biosynthetic metabolism in plant cells, it is reasonable to predict delicately balanced mechanisms for signaling and regulatory control exist in plastids. As sessile organisms, plants must be prepared to muster a panacea of responses to biotic and abiotic stresses and coordinate growth and development. Amino acid metabolism in plants, like animals, has implications beyond the production of proteins. The synthetic and catabolic pathways of the sulfur amino acids, cysteine and methionine, in particular are intertwined in numerous and vital cellular homeostatic processes. Given this level of complexity, attempts to manipulate accumulation of sulfur

metabolites in plant cells should include a careful consideration of potential ancillary effects.

GENERAL DISCUSSION

As with many advances in technology, genetic transformation of land plants has been met with mixed acceptance by the public and regulatory bodies both in the US and around the globe. Ultimately it is the potential benefit to society that must be weighed against trepidation. In the face of remonstrance, the most compelling and convincing assurances must be offered, and delivered, to overcome the many challenges that lie ahead in terms of global alimentation. Where food security and human health are concerned transgenic plants offer an alluring platform to address inadequacies given certain caveats are recognized.

Through genetic modification of plant plastids, unlike plant nuclear genomes, transgenes can be sequestered within the target host, provided the plastome is transmitted maternally as is the case in most agronomic species (Daniell, 2002, 2007). Plastid transformation experiments in the established model land plant *N. tabacum* have demonstrated repeatedly that a panoply of foreign gene products can accumulate in this setting (Verma et al., 2008). As plastid technology matures to encompass the improvement of food and feed species, a synthesis in our understanding of the mechanisms fundamental to plastid gene expression will facilitate consummation such as that which has been observed in the model system.

Toward the establishment of stable transplastomic commodity species we have investigated the use of native and foreign regulatory elements in relation to foreign gene expression in plastids. Providing support for our quantitative data on CTB-Pins protein accumulation in *L. sativa* and *N. tabacum* we have elucidated some of the basic mechanisms that may contribute to the variation we have observed. We have shown that there are multiple levels at which foreign gene expression may be regulated in our system and that these were primarily post-transcriptional. Nuclear encoded proteins with RNA binding specificity associated with cognate sequences in 5' UTRs and these sequences show marked variation across taxa suggesting that our physical data can be extrapolated beyond the model presented herein. We propose that the protein binding interaction protects the transcript and may also participate in the recruitment of accessory proteins and ultimately polyribosome assembly.

We have described the implementation of transplastomic *L. sativa* expressing the CTB-Pins fusion protein for oral delivery of an autoantigen to induce tolerance in NOD mice. Oral delivery of antigens and other human therapeutic proteins bioencapsulated in plant cells may provide a means to surmount constraints to effective drug delivery in developing nations and enhance the effective dose that reaches the gut lymphoid tissues for induction of immune responses (Limaye et al., 2006; Ruhlman et al., 2007).

Previous studies have used nuclear transgenic plants for autoantigen therapy in murine models with successful outcomes (Arakawa et al., 1998; Ma et al., 2004). Two major features of transplastomic technology make this system more desirable than the nuclear approach. First, in terms of food security, the risk of transgene escape is cause

for great concern. The potential for the introgression of pharmaceutical proteins into non-target crops that could reach livestock or consumers is simply unacceptable. Second, although foreign protein accumulation in nuclear transgenic plants has been high enough to provide efficacious protection in preclinical trials, the amount of raw plant material that would have to be ingested to deliver a relative dose to the human intestinal mucosa has precluded translation of these therapies to human subjects (Moog, 1981; Shoda et al., 2005). Gene containment *via* plastid transformation and the proven capacity for hyper-accumulation of therapeutic proteins contribute to the appeal of this technology as a pharmaceutical platform.

In the face of a pullulating global population it is imperative to identify means to produce not only greater quantities of food but also food of higher nutritional quality. Staple commodities that comprise a proportion of the diet of man and his livestock lack the essential sulfur amino acid methionine, which must not spared in the diet. To this end we generated transplastomic lines for enzymes involved in the methionine biosynthetic pathway. Using the *N. tabacum* model system we showed that CGS enzyme activity can be enhanced via plastid transformation and this led to a doubling of free methionine in leaf tissue.

In nuclear transgenic experiments where a heterologous CGS enzyme was introduced in *N. tabacum*, plants displayed severe developmental abnormalities, accumulated elevated levels of methionine catabolites and even emitted dimethylsulfide (Hacham et al., 2002; Amira et al., 2005). On the other hand similar experiments using *Medicago,* which is known to be limited in methionine content, expressed no observable

phenotype and were tremendously enhanced in free methionine (Tabe et al., 1995; Amira et al., 2005; Avraham et al., 2005). These results may suggest that the level of free methionine in *N. tabacum* may be near saturated in terms of the homeostatic balance in the plant. Our experiments with the *sat4* gene may have reflected the sensitivity of amino acid, and particularly sulfur, metabolism in plants. The use of a sulfur limited species as a platform for investigations on enhancement of methionine *via* plastid transformation could make use of the *L. sativa* system, as this species is limited in sulfur amino acid content (USDA, 1998) and may provide an amenable model to examine the efficacy of the transplastomic approach.

The application of plastid biotechnology may provide amelioration for some of the most pressing social concerns of our and future generations. We have demonstrated that transplastomic plants have the potential to accumulate foreign proteins for nutritional enhancement and for the production of biopharmaceuticals. In the interest of extending plastid transformation technology to a broader range of food and feed species we have elucidated species-specific mechanisms that regulate gene expression in plastids. It is our hope that the findings described herein will inform future studies and help bring plastid biotechnology to the consuming public as a means to enhance human productivity.

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